Stability of ultra-high temperature treated milk

The effect of raw milk quality, storage temperature and storage time

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Abstract

The composition and properties of raw milk are important for the stability of dairy products, especially for products with long shelf-life, *e.g.* ultra-high temperature (UHT) treated milk. In the UHT process, milk is subjected to temperatures above 135 °C for a few seconds, resulting in a product that can be stored for several months at ambient temperature.

This thesis investigated how natural variations in the composition and properties of raw milk affected the stability of UHT milk during long-term storage at different temperatures. For this purpose, samples of unprocessed raw milk as well as the resulting UHT milk were obtained every month for one year from a commercial dairy plant. Additionally, in a full factorial experiment, milk was modified with respect to levels of calcium, citrate and urea to investigate the effect on the stability of the resulting UHT milk.

The quality traits analysed in the raw milk included composition, bacterial counts, enzymatic activity, colour and measures of stability, *i.e.* heat coagulation time (HCT) and alcohol test. During storage of UHT milk, sensory attributes and stability measures were analysed. Multivariate statistics was used to identify batches of milk showing similarities with respect to total variation.

There were only small natural variations in the composition and properties of raw milk. Elevated calcium content, and associated decrease in pH, had a strong negative effect on the stability of UHT milk. Higher concentrations of citrate and urea in milk did not affect the stability compared with unmodified reference UHT milk.

Storage temperature correlated with colour and pH, whereas storage time correlated with creaming, sedimentation and HCT. Cold (4 °C) or ambient (20 °C) storage gave the longest shelf-life, limited by sedimentation and deviating taste. In UHT milk stored at 30 or 37 °C, the shelf-life was limited by several parameters, including colour, taste and sedimentation. Changes in the stability of UHT milk during storage are suggested to be explained by known mechanism

Knowledge of how the composition and properties of raw milk affect the stability of the resulting product can aid the dairy industry to more accurately predict the shelf-life of UHT milk. Better understanding of how storage conditions affect the shelf-life of UHT milk can provide useful guidance to consumers on appropriate storage temperature.

Keywords: raw milk, UHT milk, shelf-life, long-term storage, colour, sedimentation, creaming, heat coagulation time, ethanol stability

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Stabilitet hos mjölk med extra lång hållbarhet. Effekt av mjölkråvara, lagringstemperatur och lagringstid

Sammanfattning

Mjölkråvarans sammansättning och egenskaper är viktiga för mjölkprodukternas stabilitet och hållbarhet, särskilt för produkter med lång hållbarhet, t.ex. ultra-hög-temperatur (UHT) behandlad mjölk. I UHT-processen värms mjölk till över 135 °C i några sekunder, vilket resulterar i en produkt med en hållbarhet på flera månader vid förvaring i rumstemperatur.

I denna avhandling undersöktes hur naturliga variationer i mjölkråvarans sammansättning och egenskaper påverkade stabiliteten hos UHT-mjölk under långtidslagring vid olika temperaturer. Under ett helt år erhölls varje månad färska prover på mjölkråvaran samt den resulterande UHT-mjölken från ett mejeri. I en experimentell studie undersöktes även effekten av att modifiera mjölkråvaran avseende nivåerna av kalcium, citrat och urea på stabiliteten hos den resulterande UHT-mjölken.

Mjölkråvaran analyserades bl.a. med avseende på sammansättning, bakterier, enzymatisk aktivitet, färg och stabilitet, dvs. värmestabilitet (HCT) och alkoholtest. Under lagringen av UHT-mjölken analyserades sensoriska attribut och stabilitet. Multivariata statistiska metoder användes för att identifiera batcher av mjölk som uppvisade likheter med avseende på den totala variationen.

Vi fann endast små naturliga variationer i mjölkråvaran. Modifierad råvara med mer kalcium och lägre pH hade en stark negativ effekt på UHT-mjölkens stabilitet. En mjölkråvara med högre innehåll av citrat och urea påverkade inte stabiliteten jämfört med UHT-mjölk tillverkad av icke-modifierad mjölkråvara.

Lagringstemperatur korrelerade med färg och pH, medan lagringstid korrelerade med fettseparation, sedimentering och HCT. Förvaring i kyl (4 °C) eller rumstemperatur (20 °C) gav den längsta hållbarheten och begränsades av sedimentering och avvikande smak. Vid förvaring vid 30 eller 37 °C begränsades hållbarheten av flera parametrar, inklusive färg, smak och sedimentering. Förändringar i stabilitet under lagring av UHT-mjölken kunde förklaras med olika kända mekanismer.

Kunskap om hur sammansättningen och egenskaperna hos mjölkråvaran påverkar stabiliteten hos den resulterande produkten kan hjälpa mejeriindustrin att förutsäga hållbarheten på UHT-mjölk. Ökad förståelse för lagringsförhållandenas betydelse för UHT-mjölkens hållbarhet kan ge konsumenter vägledning om lämplig förvaringstemperatur.

Nyckelord: mjölkråvara, UHT-mjölk, bäst-före-datum, lagring, färg, sedimentbildning, fettseparation, värmestabilitet, etanolstabilitet

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It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of light, it was the season of darkness, it was the spring of hope, it was the winter of despair.

Charles Dickens, A Tale of Two Cities

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Karlsson, M.A.***, Langton, M., Innings, F., Wikström, M. & Lundh, Å.S. (2017). Short communication: Variation in the composition and properties of Swedish raw milk for ultra-high-temperature processing. *Journal of Dairy Science*, vol. 100 (4), pp. 2582–2590. DOI: https://doi.org/10.3168/jds.2016-12185

II **Karlsson, M.A**.*, Langton, M., Innings, F., Malmgren, B., Höjer, A., Wikström, M. & Lundh, Å. (2019). Changes in stability and shelf-life of ultra-high temperature treated milk during long term storage at different temperatures. *Heliyon*, vol. 5 (9), p. e02431. DOI: https://doi.org/10.1016/j.heliyon.2019.e02431

III **Karlsson, M.A.***, Lundh, Å., Innings, F., Höjer, A., Wikström, M. & Langton, M. (2019). The Effect of Calcium, Citrate, and Urea on the Stability of Ultra-High Temperature Treated Milk: A Full Factorial Designed Study. *Foods*, vol. 8 (9), p. 418. DOI: https://doi.org/10.3390/foods8090418

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The contribution of Maria Karlsson to the papers included in this thesis was as follows:

I Participated in planning the study together with the supervisors and industrial partners, carried out most of the experimental work, and had main responsibility for evaluating the results and for writing and revising the manuscript.

II Had main responsibility for planning the study together with the supervisors and industrial partners, carried out the experimental work and the statistical analysis, had main responsibility for evaluating the results, and was responsible for writing and revising the manuscript.

III Participated in planning the study together with the supervisors and industrial partners, carried out the experimental work, performed the statistical analysis, and had main responsibility for evaluating the results and for writing and revising the manuscript.

Abbreviations

ANOVA	Analysis of variance
CFU	Colony forming units
DoE	Design of experiments
HCT	Heat coagulation time
PCA	Principal component analysis
PLS	Partial least square
SCC	Somatic cell count
SLU	Swedish University of Agricultural Sciences
TBA	Tetra Brik Aseptic
UHT	Ultra-high temperature

1 Introduction

Milk and dairy products have been a part of the human diet since ancient times. Milk is an excellent source of many essential nutrients and in many parts of the world consumption of dairy products is still part of the food culture. Besides being a nutrient-rich food, milk is an excellent growth medium for microorganisms. Therefore a milestone in the history of dairy production was in the late 19th century, when Louis Pasteur experimented with heating methods and invented pasteurisation, a method that laid the foundation for hygienic processing by significantly decreasing the number of spoilage microorganisms in milk, thereby extending the shelf-life. Since 1937, Swedish legislation states that milk marketed for consumption must be pasteurised (LIVSFS 2005:20).

For manufacture of all dairy products, the composition and properties of the raw milk are important, affecting the stability and shelf-life of the product. Besides the gradual long-term changes in raw milk composition, day-to-day variations in raw milk quality constitute a challenge for the dairy industry (Chen *et al.*, 2014).

There are generally two approaches in studies investigating how variations in raw milk composition correlate with product stability. These are: studying the effect of natural variations in milk composition and studying the effects of modifying the milk (Williams, 2002). Both approaches were used in this thesis work. Papers I and II investigated how the natural variation in raw milk affected the stability of the resulting ultra-high temperature (UHT) treated milk. In Paper III, a full factorial experiment was conducted to investigate how modification of the raw milk affected the stability of the resulting UHT milk.

1.1 Raw milk quality and composition

Raw milk quality, routinely measured at the farm and at the dairy plant, can be defined from various perspectives. For instance, raw milk quality can be linked to the milk payment system, in which the price paid to dairy farmers is related to protein and fat content, somatic cell count (SCC) and microbiological quality (Arla Foods, 2019; Mortensen *et al.*, 2010). Alternatively, raw milk quality can be defined as milk of normal colour, free of off-flavours and free of foreign substances (*e.g.* antibiotics, biocides, detergents) (Murphy *et al.*, 2016).

1.1.1 Factors affecting raw milk quality

Cow's milk produced in Sweden typically contains 86.6% water, 4.7% carbohydrates, 4.2% fat, 3.5% protein and 1% minerals and vitamins (Lindmark-Månsson, 2012; Växa Sverige, 2019) (*Figure 1*). In general, natural variations in raw milk composition can be attributed to factors related to the animal or to the animal's environment. Factors suggested to explain variations in raw milk composition are season, stage of lactation, feed, breed, animal health and management regime (Chen *et al.*, 2014; Fox & McSweeney, 1998; Heck *et al.*, 2009; Williams, 2002).

Milk quality traits reported to vary with season include protein and fat content (Heck *et al.*, 2009; Lindmark-Månsson *et al.*, 2003) and mineral content (Sola-Larrañaga & Navarro-Blasco, 2009), but also a number of other traits (Gaucher *et al.*, 2008a).

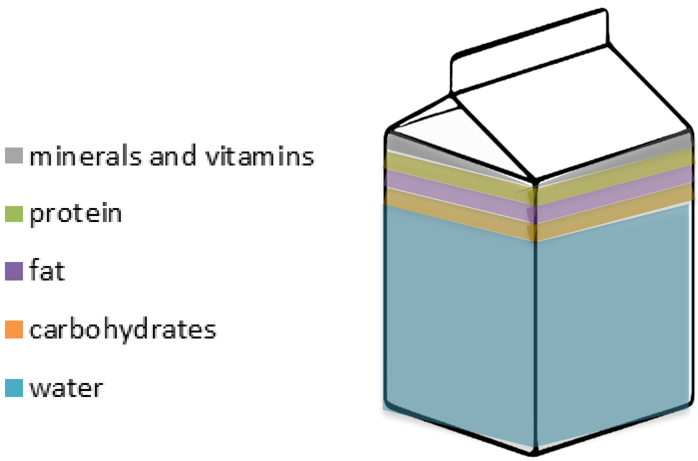


Figure 1. Cow's milk contains water, carbohydrates, fat, protein, minerals and vitamins.

In Sweden, calving patterns are non-seasonal, resulting in bulk milk from cows at various stages of lactation all year round (Jordbruksverket, 2012). This reduces the seasonal variation in raw milk composition, but does not entirely eliminate it (Auldist *et al.*, 1998). Stage of lactation affects raw milk composition, *e.g.* a high yield of milk with low protein and fat content and high citrate content is reported in early lactation (Garnsworthy *et al.*, 2006; Palladino *et al.*, 2010).

In addition to stage of lactation, feed is a major factor contributing to variations in raw milk quality. In general, Swedish dairy cows are fed 70% silage and 30% cereal concentrate and, under Swedish animal welfare legislation, all cows must be allowed grazing outdoors for a minimum of two months during summer (Jordbruksverket, 2019a).

Swedish Holstein and Swedish Red and White are the two dominant breeds in Sweden, in 2018 representing approximately 56% and 35% of dairy cows, respectively (Växa Sverige, 2019) (*Figure 2*). The Swedish Holstein breed is known for high milk yield, whereas Swedish Red and White has a lower yield of milk, but with a higher protein and fat content.

The association between herd size, animal health and welfare is complex and affected by many factors, including various aspects of management (Barkema *et al.*, 2015). In all developed countries, the number of dairy farms has decreased in recent decades and animal herd size has increased (Barkema *et al.*, 2015). The average herd in Sweden has 92 cows, with herds generally

Figure 2. Milking of Swedish Holstein and Swedish Red and White at the Swedish Livestock Research Centre at SLU, Photo: Jenny Svennås-Gillner, SLU.

being smaller in northern Sweden and larger in the south-west (Växa Sverige, 2019). In Sweden, the percentage of small tie-stalls herds has decreased, in favour of larger free-stalled herds served by automated milking systems (Jordbruksverket, 2019b). Studies comparing different management systems, *i.e.* automated and conventional milking systems, have found that bulk milk samples from automated systems have a lower protein and plasmin content and higher SCC and total proteolysis (Johansson *et al.*, 2017).

The composition of raw milk delivered to Swedish dairies was investigated in 2001 and again in 2009 (Lindmark-Månsson, 2012; Lindmark-Månsson *et al.*, 2003), and more recently in Paper I. Comparison of the data obtained on the composition of raw milk shows that there have been several changes over time that can be expected to have a negative impact on the stability of UHT milk, *e.g.* a 5% increase in calcium content, a 20% decrease in citrate content and a 7% decrease in urea content between 2001 and 2009.

1.1.2 The effect of calcium, citrate and urea in milk

Calcium is important for the internal structure and stability of the casein micelle, both via the colloidal calcium phosphate associated to casein molecules and the formation of calcium bridges between negatively charged residues of the caseins (Gaucheron, 2005; Tsioulpas *et al.*, 2007). In milk serum, calcium forms complexes with other agents or exists as free ions. Ionic calcium has been shown to vary considerably between cows (Tsioulpas *et al.*, 2007; White & Davies, 1958). The calcium equilibrium between the colloidal and serum phase is also affected by pH and temperature, whereby a reduction in pH or decrease in storage temperature increases the calcium concentration in the milk serum (Gaucheron, 2005; Lewis *et al.*, 2011; Tsioulpas *et al.*, 2007). Total calcium content in milk is reported to be on average 26-32 mM *(*Chen *et al.*, 2014; Walstra *et al.*, 2006).

Citrate, mainly present in milk serum, plays an important role in the mineral equilibrium of milk and is part of a buffering system between calcium and hydrogen ions. In appropriate concentrations, citrate improves the stability of the milk by forming soluble complexes with calcium ions, preventing precipitation of calcium phosphate (Whittier, 1929). With higher buffering capacity, it is suggested that milk is subjected to less variation in pH as its temperature increases, which may contribute to higher heat stability (Salaün *et al.*, 2005). High levels of citrate have been shown to increase calcium levels in milk serum and consequently decrease the colloidal calcium phosphate content in casein micelles (Gaucheron, 2005). The average content of citrate in milk is 7-11 mM (Walstra *et al.*, 2006). Some studies have found that citrate content in milk varies significantly with feed and stage of lactation (Faulkner & Peaker, 1982; Garnsworthy *et al.*, 2006), whereas other studies have found no correlation to feed (Akkerman *et al.*, 2019).

A higher concentration of urea in milk is reported to act stabilising on the micelle (Williams, 2002), possibly by preventing protein cross-linking and aggregation (Deeth & Lewis, 2016). Crowley *et al.* (2014) suggest that at pH ≥6.8 urea degraded to ammonia has a buffering and stabilising effect on milk protein concentrate suspensions, observed as longer heat coagulation time. Lindmark-Månsson (2012) reported an average urea level of 4.7 mM in Swedish milk. Milk urea is known to vary with stage of lactation, season and the protein content of the feed (Auldist *et al.*, 1998; Carlsson *et al.*, 1995; Reid *et al.*, 2015).

1.2 Ultra-high temperature processing

Early in the 20th century, UHT processing was developed. In UHT treatment, milk is subjected to high temperatures, above 135 °C, for a few seconds, resulting in a product with a shelf-life of several months when stored at ambient temperature. Since UHT milk does not require cold storage and has a long shelf-life, it offers a convenient way of providing high quality milk to consumers across the world, including those in regions without cold chain infrastructure.

Direct and indirect UHT treatment are currently the two main types of system available on the market. In direct systems, the heating medium, *e.g.* hot steam, is in direct contact with the milk, whereas in indirect systems heat is transferred via a heat exchanger.

It was not until Tetra Pak introduced aseptic packaging in 1961 that UHT milk became commercially available (Tetra Pak, 2019c). In aseptic processing, the product and the package are sterilised separately, then combined and sealed in a sterile atmosphere. By applying this processing and packaging technique, recontamination of the product is prevented, resulting in a product with a long shelf-life. Ever since the introduction of UHT treated products, the processing efficiency and product quality have improved (Deeth, 2010).

1.2.1 Consumer preferences for UHT milk

Availability and preference for pasteurised or UHT treated milk vary between countries. The sensory perception and appreciation of milk varies greatly among consumers (Walstra *et al.*, 2006), and is mainly affected by the fat, protein and lactose content and by the manufacturing process (Oupadissakoon *et al.*, 2009; Schiano *et al.*, 2017).

Compared with pasteurised milk, UHT milk has more cooked and sulphuric flavours, due to the extreme heating (Schiano *et al.*, 2017). These flavours are generally regarded as undesirable by Swedish consumers, which is one of the reasons why UHT milk only has a limited market share in Sweden. In global terms, UHT milk is an important dairy product and the demand for UHT milk and UHT milk-based products is increasing (Deeth & Lewis, 2017). With the increasing production of novel UHT milk-based beverages, variation in product stability has led to a growing interest in the effect of raw milk quality on UHT products during storage. This issue was the main focus of this thesis work.

1.3 Factors affecting the stability and shelf-life of UHT milk during storage

The stability of a food gradually changes over time, with impacts on the product's shelf-life. Shelf-life is the period of time in which food products are stable and viable for consumption (Tetra Pak, 2019b). A common definition of shelf-life is the time during which the food product will:

- remain safe
- retain desired sensory, chemical, physical and microbiological characteristics
- maintain a composition that complies with the label declaration, when stored under the recommended conditions (Tetra Pak, 2019b).

Although UHT milk is commercially sterile, it is not possible to destroy all bacteria and inactivate all heat-resistant enzymes and thereby prevent chemical and physical, and sometimes enzymatic, reactions from taking place and changing the quality attributes of the product (Deeth & Lewis, 2017). In milk of high hygienic standard, chemical and physical changes during storage are the most important factors limiting the shelf-life (Mortensen *et al.*, 2010). These changes include *e.g.* proteolytic, lipolytic, oxidative and Maillard type reactions (Singh *et al.*, 2009; van Boekel, 1998). Chemical and physical changes in the milk can lead to fat separation (creaming), sediment formation, gelation, browning or development of off-flavours during subsequent storage (Anema, 2017; Deeth, 2010). Higher storage temperature generally leads to faster loss of stability, with the exception of solubilisation of caseins and colloidal calcium phosphate, a process that is faster at lower storage temperatures. Other factors affecting the speed at which the stability changes during storage include *e.g.* heat load during UHT processing, dissolved oxygen availability, milk composition and activity of heat-resistant enzymes.

1.3.1 Enzymatic reactions

In milk, enzymes can either be endogenous or exogenous, *i.e.* of bacterial origin. Endogenous enzymes can either enter the milk from the blood or originate from the somatic cells (Le *et al.*, 2017). High numbers of somatic cell counts and psychrotrophic bacteria in raw milk are related to the occurrence of enzymatic activity, damaging milk components and with potential negative impacts on the shelf-life of UHT milk (Murphy *et al.*, 2016).

Monitoring spoilage microorganisms which produce enzymes that may give rise to product deterioration during storage and reduce shelf-life is important in the dairy industry (Deeth & Lewis, 2017). In general, raw milk bacterial counts need to exceed 1 000 000 colony forming units (cfu) per mL to cause defects in UHT milk during storage (Murphy *et al.*, 2016). The major psychrotrophic bacteria in raw milk is *Pseudomonas* species (Deeth & Lewis, 2017). Some species of *Pseudomonas* can produce heat-resistant enzymes that remain active in the milk after UHT processing, causing proteolysis and lipolysis during storage. Griffiths (1986) found that 20-40% of *Pseudomonas* proteases remained active after heating at 140 °C for 5 seconds.

Proteolysis

The importance of the endogenous enzyme plasmin was studied by Rauh *et al.* (2014b), who suggested that 75% of the proteolytic products found in milk could derive from plasmin activity. Plasmin-induced hydrolysis of β-casein, resulting in γ-casein, proteose-peptone and smaller peptides, is strongly correlated with storage temperature *(Mortensen et al.*, 2010). Plasmin is inactivated by indirect UHT treatment (Manji *et al.*, 1986). Proteolysis in UHT milk is suggested to be involved in the development of bitter flavour and age gelation (Rauh *et al.*, 2014a).

Lipolysis

Lipolysis is a reaction in which lipases hydrolyse triacylglycerides to free fatty acids, resulting in rancid off-flavours. The endogenous lipoprotein lipase is heat labile and is inactivated by the UHT process (Kong & Singh, 2017). However, some bacterial lipases can survive the UHT treatment (Panfil-Kuncewicz *et al.*, 2005). Indirect UHT treatment more effectively inactivates lipases than direct UHT systems (Panfil-Kuncewicz *et al.*, 2005). Disrupting the milk fat globule membrane, *e.g.* by homogenisation, exposes the triacylglycerides, making them accessible for lipases, and increases the rate of lipolysis. It has been suggested that lipolysis in milk may increase with modern automated milking systems where the milk is pumped in small amounts, with a high air content and often over long distances (Mortensen *et al.*, 2010). Raw milk with free fatty acid levels exceeding >1.0 mmol/L should not be used for UHT processing, as this would increase the risk of causing rancid flavour in the product.

1.3.2 Oxidation

Pronounced oxidation of lipids or proteins leads to oxidised off-flavours in milk and dairy products, caused by lipid-derived carbonyls and the derived alcohols or oxidation of amino acids and proteins (Mortensen *et al.*, 2010). Specifically, oxidation of phospholipids during long-term storage can produce a cardboard-like flavour (Walstra *et al.*, 2006). Oxidation depends on the concentration of dissolved oxygen in the milk immediately after processing and on the storage temperature (Jensen *et al.*, 2015; Kong & Singh, 2017).

1.3.3 Stokes' law

According to Stokes' law, the terminal velocity at which a particle moves through a liquid is directly proportional to the size and density of the particle. During storage of UHT milk, the effect of Stokes' law may be observed as particles either floating to the top or sinking to the bottom of the package under the influence of their own weight (Dalgleish, 1992). This results in fat separation, fat adhesion to the packaging material or sediment formation (Ramsey & Swartzel, 1984). The rate of reaction is affected by particle size, with faster movement of larger particles, and has also been shown to increase with storage temperature (Hardham *et al.*, 2000; C. Lu *et al.*, 2013; Ramsey & Swartzel, 1984). Fat separation is also closely correlated with, and increases with, fat content. In addition, fat separation is affected by the homogenisation efficiency, where higher efficiency retards fat separation by contributing to a larger reduction in fat globule size (Lu *et al.*, 2013).

In UHT products, sediment formation is a well-known problem. The sediment, forming a compact non-dispersible layer adhering to the bottom of the package, is suggested to consist of aggregates of proteins or protein particles of various sizes (Gaur *et al.*, 2018; Malmgren *et al.*, 2017). Aggregation of micelles and increased storage temperature increase the rate of sedimentation (Dalgleish, 1992; Gaur *et al.*, 2018; Malmgren *et al.*, 2017;

Ramsey & Swartzel, 1984). Recent studies have found that sediment formed after eight weeks of storage is depleted in κ-casein and has low levels of α-lactalbumin and β-lactoglobulin (Gaur *et al.*, 2018; Malmgren *et al.*, 2017).

1.3.4 Maillard reaction

Formation of flavour compounds and brown pigments

Changes in the flavour and colour of UHT milk during storage can be explained by the Maillard reaction, which results in the formation of brown pigments and caramelised flavours (Schiano *et al.*, 2017; van Boekel, 1998). The Maillard reaction is known to increase with storage temperature. In the initial stages of the reaction, the reducing sugar in milk (mainly lactose) condenses with amino groups (mainly lysine residues), a reaction called lactosylation, to form the so-called Amadori compound (van Boekel, 1998). Volatile compounds are mainly formed in the intermediate stage (Sunds *et al.*, 2018). In the final stage of the Maillard reaction, a non-enzymatic browning reaction consisting of condensation of amino compounds and sugar fragments, brown melanoidins and intra-micellar protein cross-links are eventually formed (Al-Saadi & Deeth, 2008; van Boekel, 1998).

Effect on pH

In the complex Maillard reaction, lactose is subjected to isomerisation and degradation, creating significant amounts of formic acid that lower the pH (van Boekel, 1998). As the pH decreases, the negative net charge on proteins is reduced, promoting inter-micellar interactions (Day *et al.*, 2017).

The heat coagulation time (HCT) of milk has been shown to depend on the pH, calcium ion concentration and casein micelle size, whereby a small reduction in pH significantly decreases the HCT (Deeth & Lewis, 2017; Singh, 2004; van Boekel *et al.*, 1989). The HCT can be used to predict the heat stability of raw milk by determination of the time it takes for milk to visibly coagulate when heated to temperatures above 100 °C (Davies & White, 1966). High heat stability of milk is suggested to minimise fouling during the UHT process and sediment formation in the product (Deeth & Lewis, 2016). However, the correlation between HCT and the stability of milk for commercial processing has been questioned (H. Singh, 2004), due to the fact that the heating profiles used in HCT determination and in UHT treatment are different.

1.3.5 Protein cross-linking

The term ‘protein cross-linking’ can describe the covalent bonding of proteins within a casein micelle (intra-micellar) or between micelles (inter-micellar). Both intra- and inter-micellar cross-links are suggested to increase with storage temperature and storage time. For instance, Andrews (1975) found that in UHT milk stored at 4 °C for 9 months only minor intra-micellar protein cross-linking occurred, whereas in UHT milk stored at 37 °C >50% of the caseins were polymerised or cross-linked. Al-Saadi and Deeth (2008) and Holland *et al.* (2011) observed an increase in high molecular weight proteins in UHT milk stored at temperatures >20 °C.

Age gelation, which is commonly observed in UHT milk, results in a three-dimensional voluminous inter-micellar linked network of proteins (Anema, 2017). In general, gelation occurs when casein micelles lose colloidal stability, preceded by changes at the surface of the casein micelles (Datta & Deeth, 2001). Intra-micellar protein cross-links, formed as part of the Maillard reaction, are suggested to provide protection against age gelation by preventing dissociation of β-lactoglobulin-κ-complexes from the micelles (McMahon, 1996). It is also suggested that lactosylation, part of the Maillard reaction including blocking of the reactive lysine residues, prevents micelles from interaction and form a gel (Malmgren *et al.*, 2017). Indirect UHT treatment or a higher heat load during processing delays gel formation, with more severe heating increasing the level of denatured whey proteins forming complexes with caseins, but also resulting in greater inactivation of enzymes, delaying the onset of gelation (Manji *et al.*, 1986).

Increased intra-molecular cross-linking between κ-casein and β-casein has been shown to correlate with increased ethanol stability in raw milk (Huppertz & de Kruif, 2007). In contrast, it has been suggested that factors reducing the negative net charge of the casein micelle, *e.g.* low pH caused by acid-producing bacteria, salt imbalance, high concentration of ionic calcium or high amount of serum proteins (*e.g.* in colostrum), may contribute to reduced ethanol stability (Horne, 2016; Horne & Parker, 1981; Lewis *et al.*, 2011). The ethanol stability test, also called the alcohol test, has been used for over a century as a simple, cheap and quick pass-fail test to detect poor quality raw milk that is not suitable for UHT processing (Horne, 2016). In milk, failing the ethanol stability test, aggregates of inter-micellar linked proteins can be observed. It is recommended that raw milk for UHT processing has an ethanol stability of ≥74% (Shew, 1981), generally corresponding to a calcium ion concentrations <2.0 mM (Tsioulpas *et al.*, 2007). Deeth and Lewis (2017) suggest that ethanol stability, pH and ionic calcium should be routinely measured in raw milk to avoid problems related to fouling or sediment formation in UHT milk.

1.3.6 Deamidation

During storage of UHT milk, significant non-enzymatic deamidation occurs. Deamidation involves hydrolysis of the amide groups in the amino acid asparagine or in glutamine residues of proteins to aspartic acid and glutamic acid and ammonia, resulting in proteins with increased electrostatic repulsion between caseins due to higher negative net charge (Broyard & Gaucheron, 2015; Holland *et al.*, 2011). Deamidation increases with storage temperature.

Table 1. *Summary of the main events occurring in ultra-high temperature treated milk during storage that affect the stability*

Mechanism	Effect on stability during storage
Enzymatic activity	Degradation of proteins or fat by proteolysis, lipolysis or oxidation can result in off-flavours. Proteolysis is also suggested to contribute to gel formation. The rate of enzymatic reactions increases with storage temperature.
Oxidation	Oxidation of fat give rise to aldehydes, ketones and alcohols, perceived as rancid off-flavour. The rate of reaction increases with storage temperature, available oxygen, lower water activity and lower levels of antioxidants.
Stokes' law	According to Stokes' law, particles either rise to the top, resulting in fat separation, or settle, forming a compact layer adhering to the bottom of the package, an effect often referred to as sediment formation. The rate of reaction increases with storage temperature.
Maillard reaction	A non-enzymatic browning reaction in which amino acids and reducing sugars condense, affecting the colour and taste of the milk. The rate of reaction increases with storage temperature.
Protein cross-linking	Intra-micellar protein cross-linking occurs as part of the advanced Maillard reaction. Intra-micellar cross-links between caseins can contribute to an increased negative net charge on the micelles. The result of inter-micellar cross-links is visually seen in samples failing the heat coagulation test or the alcohol test. Inter-micellar linked proteins are found in age gelled milk. Intra- and inter-micellar cross-links increase with storage temperature.
Deamidation	A non-enzymatic reaction resulting in proteins with higher negative charge. Deamidation increases with storage temperature.

2 Objectives and hypotheses

The overall aim of this thesis work was to investigate how variations in the composition and properties of raw milk affect the stability of UHT milk during storage at different temperatures. The possibility to predict the stability of the UHT milk using various markers was also investigated.

Specific objectives and hypotheses were:

- to investigate the natural variation in Swedish raw milk collected monthly during one year and determine whether variations persist after UHT processing. The hypothesis was that variations in raw milk composition between months and differences between sampling months persist after UHT processing (Paper I).

- to investigate the shelf-life of UHT milk when stored at different temperatures for up to one year after production and identify the factors that limit the shelf-life of the product. The hypothesis was that the shelf-life of UHT milk is significantly affected by storage temperature and is limited by gel formation, sedimentation, a deviating taste or colour (Paper II).

- to evaluate, in a full-factorial design experiment, the effect of calcium, citrate and urea and their interactions on the stability of UHT milk stored at different temperatures for up to one year after production. The hypothesis was that an increasing level of calcium has a negative impact on the stability of UHT milk and that an increasing level of citrate or urea has a positive impact on the stability, resulting in longer shelf-life (Paper III).

3 Materials and methods

This chapter describes sample collection, storage conditions and methods used to evaluate the quality of raw milk and the stability and shelf-life of UHT milk in long-term storage trials. It also describes the modification of raw milk, and the effects on the corresponding UHT milk, investigated in a full factorial experiment.

3.1 Collection and preparation of milk samples

Different types of milk samples were used in two long-term storage trials. In the first trial, studying natural variations in the raw milk, the samples were obtained from a commercial dairy plant in Luleå (Norrmejerier). In the second trial, using modified milk, the samples were processed at a pilot plant facility in Lund (Tetra Pak Product Development Centre).

Commercial dairy plant

In total, 11 batches of raw dairy silo milk and 11 batches of the resulting UHT milk were sampled on a monthly basis for one year (December 2014-November 2015), with the exception of August, as described in Paper I. The silo milk consisted of batches of raw milk received from approximately 80 dairy farms located in the region surrounding the dairy plant.

A representative raw milk sample was collected and transported refrigerated to the Swedish University of Agricultural Sciences (SLU), Uppsala. Analysis of the raw milk started on the day after arrival at SLU and all raw milk samples were analysed with four days of milking, including cold storage on farms and at the dairy plant. Raw milk samples were aliquoted and frozen at -20 °C for later analysis of protein profile, mineral, citric acid and urea content and of enzymatic activity.

Pilot plant facility

Based on previous studies reporting significant changes in Swedish raw milk composition, at the pilot plant facility milk was modified with respect to levels of calcium, citrate and urea, to study how this would affect the stability of the UHT milk.

The experiment was set up as a 2^3 full factorial design, as described in Paper III. In total, nine batches of milk were prepared, for practical reasons on two different occasions (November 2014 and February 2015). The nine batches of UHT milk differed with respect to levels of calcium, citrate and urea in different combinations, and also included two batches of unmodified reference milk (*Table 2*). Based on previously reported average levels of calcium, citrate and urea in milk of 32, 9 and 4.7 mM, respectively (Lindmark-Månsson, 2012; Walstra *et al.*, 2006), modified milk batches containing 20% higher concentrations of the different components were prepared. Calcium ($CaCl_2$ * 2 H_2O, VWR Chemicals, Leuwen, Belgium), citrate ($Na_3C_6H_5O_7$ * $2H_2O$, VWR Chemicals, Leuwen, Belgium) and urea ($(CO(NH_2)^2$, Alfa Aesar, Karlsruhe, Germany) were dissolved in approximately 200 mL distilled water and added to batches of 200 L pasteurised, standardised (1.5% fat) and homogenised milk, to obtain calculated final concentrations of 38 mM calcium, 11 mM citrate and 5.6 mM urea in the milk. The modified milk was stored refrigerated overnight and subjected to UHT processing on the following day. Directly before UHT processing, the milk was analysed for total solids, lactose, fat and protein content, as well as colour, heat coagulation time, ethanol stability and freezing point, according to the methods described in section 3.3.

Table 2. *Experimental design of the 2^3 full factorial designed study at the pilot plant facility. Batches of milk with elevated levels of calcium, citrate and urea were prepared in the combinations shown and subjected to ultra-high temperature treatment. Batches 1-5 and 6-9 were produced on different occasions, with one unmodified reference batch (batch 5, 6) included on each occasion. Abbreviations: +20% = elevated level; - = normal level*

Batch	Calcium	Citrate	Urea
1	+20%	-	-
2	-	+20%	-
3	-	-	+20%
4	+20%	+20%	+20%
5	-	-	-
6	-	-	-
7	+20%	+20%	-
8	+20%	-	+20%
9	-	+20%	+20%

3.2 Ultra-high temperature processing and storage

At the commercial dairy and at the pilot plant, the milk was standardised to 1.5% fat, pre-heated to 82 °C, de-aerated, homogenised in two stages (150 + 30 bar) and UHT treated using indirect tubular heat exchangers at 137 °C for 4 seconds. The UHT milk was aseptically packed in 1-L (dairy plant) or 250-mL (pilot plant) Tetra Brik Aseptic (TBA) packages.

The UHT milk was transported at ambient temperature to SLU, Uppsala (*Figure 3*). A fresh UHT milk sample, no older than 1-2 weeks, was analysed on the day after delivery to SLU, and thereafter the UHT milk was stored at 4, 20, 30 or 37 °C for up to 52 weeks. Every month during storage, packages of UHT milk were removed, stored at room temperature overnight and analysed on the following morning. All analyses were performed at ambient temperature unless otherwise stated. Sub-samples of UHT milk samples were then frozen at -20 °C for later thawing and analysis of mineral, citric acid and urea content, and enzymatic activity.

Figure 3. (Left) Every month for one year, 144 1-L Tetra Brik Aseptic packages of ultra-high temperature (UHT) milk were delivered from Norrmejerier's commercial dairy plant in Luleå to the Swedish University of Agricultural Sciences (SLU). (Right) On two occasions during the year, UHT milk processed at the pilot plant facility at Tetra Pak Product Development Centre in Lund and packed in 250-mL packages was delivered to SLU.

3.3 Characterisation of milk samples

3.3.1 Total solids, protein, fat, lactose, somatic cell count and freezing point

The content of total solids, protein, fat and lactose in milk was measured by near-infrared spectroscopy, using MilkoScan FT120 (Foss, Hillerød, Denmark). The protein profile of the raw milk was analysed by capillary electrophoresis, as previously described by Gustavsson *et al.* (2014). Somatic cell count was measured using Fossomatic FC (Foss, Hillerød, Denmark). The freezing point of the raw milk samples was estimated using MilkoScan FT2 (Foss, Hillerød, Denmark).

3.3.2 Citrate, urea and minerals

Citrate content was analysed by high-performance liquid chromatography (HPLC) according to the method described by Andersson and Hedlund (1983). Urea content was determined using the AutoAnalyzer III procedure (SEAL Analytical GmbH, Norderstedt, Germany) according to Eriksson and Rustas (2014). Calcium, potassium, sodium and magnesium content were analysed as described in ISO 8070 (ISO/IDF, 2007) using atomic absorption spectrometry (AAnalyst 100, Perkin Elmer, Waltham, MA, USA).

3.3.3 Total bacterial count and psychrotrophic microorganisms

In this thesis, counts of total bacteria and psychrotrophic microorganisms were enumerated in raw milk samples with colony count techniques, according to the NMKL method 86 (NMKL, 1999) and ISO 8552 (ISO/IDF, 2004), respectively.

3.3.4 Total proteolysis, plasmin and plasminogen derived activity

Total proteolysis in the raw milk and in fresh UHT milk from the commercial dairy plant was measured based on the reaction of primary amino groups of trichloroacetic acid-soluble peptides and free amino acids with fluorescamine (Wiking *et al.*, 2002). Equal volumes of milk and 24% trichloroacetic acid were mixed, held on ice for 30 min, and centrifuged at 16 000 × *g* for 20 min at 4 °C (Himac CT15RE, Hitachi Koki Co., Ltd., Tokyo, Japan). The supernatant was mixed with sodium tetraborate and fluorescamine, and the fluorescence (excitation wavelength 390 nm, emission wavelength 480 nm) was measured after 18 min in a luminescence spectrometer (LS 55, Perkin Elmer).

Plasmin activity and plasminogen-derived activity were measured in the raw milk and fresh UHT milk according to the method of Korycka-Dahl *et al.* (1983), with modifications described by de Vries *et al.* (2016). Total activity was obtained by activation of plasminogen into plasmin by addition of urokinase and plasminogen-derived activity was calculated as the difference between total activity and plasmin activity. Activity was expressed as U/mL in all cases, with one unit (U) defined as the amount of enzyme that produces a $\Delta A405$ of 0.001 per min at 37 °C due to *p*-nitroanilide released from the chromogenic substrate.

3.3.5 Heat coagulation time, pH and ethanol stability

In this thesis, HCT, pH and ethanol stability were measured on raw milk, but also on the UHT milk, to see whether the results could give additional information explaining observed changes in stability of the processed milk during storage at different temperatures. Heat coagulation time was measured as described in Paper I. In brief, HCT was defined as the time needed for visual coagulation of 0.5 mL milk in a sealed test-tube rocked at 130 °C (Davies & White, 1966) using dedicated equipment from Hettich Benelux (Geldermalsen, Netherlands).

In all studies, the pH was measured using an IoLine electrode (SI Analytics®, Mainz, Germany).

Ethanol stability was defined as the highest ethanol concentration that could be added to the sample without causing visual coagulation of the milk. Equal volumes of milk and ethanol, at ethanol concentrations ranging between 40 and 100% in 2% increments, were mixed in an Eppendorf tube and incubated at room temperature for 30 min before evaluation (White & Davies, 1958).

3.4 Evaluation of product stability

3.4.1 Fat separation and fat adhesion

To evaluate fat separation, defined as the thickness of the cream layer floating on the surface, the flaps on the TBA package were turned up, the top of the package cut off and the thickness of the cream layer was rated on a four-point scale: no visual cream layer, waves of cream, surface completely covered with fat, and lumps/clots of fat (*Figure 4*). Fat adhesion, defined as the thickness and amount of fat adhering to the inside of the package after the milk had been poured out, was compared with reference photos and rated on a scale of 0-4 (Paper II), modified from a protocol of New Zealand Dairy Industry (2000a). A fat separation score of 3 or a fat adhesion of ≥3.5 was considered the threshold at which the product was no longer acceptable to consumers.

Figure 4. Examples of fat separation, ranging from (left) visible waves of cream on the package base to (right) advanced fat separation with visible lumps of fat.

3.4.2 Sediment formation and gelation

In this thesis, the amount of sediment formed at the bottom of the package was visually estimated, compared with reference photos and graded on a scale of 0-100%, where 0 corresponded to no sediment and 100% corresponded to the bottom of the package being completely covered with sediment (New Zealand Dairy Industry, 2000b; Paper II). Milk with sediment covering more than 45% of the bottom of the package was regarded as not acceptable for consumption.

Samples were visually inspected for absence or presence of age gelation.

3.4.3 Taste and colour

Sensory evaluation, including tasting the milk, was performed by a trained person from storage weeks 16 to 52 on UHT milk produced at the commercial dairy plant in January, March, May, July, September and November. The taste was graded as normal or with small deviation or large deviation and, if possible, the deviating taste was described in words. Taste deviations, small or large, were considered unacceptable to consumers.

Changes in colour were evaluated using CIELAB colour space and measured with a CM-600d spectrophotometer (Konica Minolta, Shanghai, China). Using this technology, L* indicates lightness ranging from 0-100, a* indicates a range from green to red (-60 to +60) and b* a range from blue to yellow (-60 to +60). In this thesis, milk with L* values <76 was considered unacceptable for consumption.

3.5 Statistical evaluations and experimental design

Multivariate analysis involves observation and analysis of more than one variable at a time, with the aim of obtaining an overview of all the information in the data set (Jolliffe, 2002).

3.5.1 Principal component analysis and correlations

Principal component analysis (PCA) is defined as an orthogonal linear transformation that transforms the data to a coordinate system such that the greatest variance by any projection of the data comes to lie on the first coordinate (principal component (PC) 1), the second greatest variance on the second coordinate (PC2) *etc.* (Jolliffe, 2002). To make the coefficients comparable when responses have different ranges, the values are normalised, *i.e.* each coefficient is divided by the standard deviation of its respective response. Normalising can mean putting different variables on a common scale, but it can also mean applying a transformation so that the transformed data roughly show a normal distribution.

In Paper I, PCA was used to identify months showing similarities with respect to the total variation, using SIMCA 13.0 software (Umetrics, Umeå, Sweden).

Minitab 18 software (Minitab Ltd, State College, Pennsylvania) was used to calculate Pearson correlation coefficient. Probability (P-) values were calculated by one-way analysis of variance (ANOVA) (two-sided 95% confidence interval).

3.5.2 Experimental design and partial least square regression analysis

Design of experiments (DoE) is one of the most valuable techniques for organised and efficient planning, execution, and statistical evaluation of experiments. The concept of DoE is to vary process parameters simultaneously over a set of planned experiments and then interpret the results by means of a proven mathematical model. When using a factorial design, it is possible to extract the main effects, comparing all values at high levels and low levels, thereby increasing the statistical power of the analysis.

In Paper III, partial least squares (PLS) regression analysis was carried out using MODDE 11 software (Umetrics, Umeå, Sweden) with the factors calcium, citrate and urea and the interaction terms [calcium x citrate], [calcium x urea], [citrate x urea] and [calcium x citrate x urea]. Response variables were fat separation, fat adhesion, sedimentation, colour, pH, ethanol stability and HCT. The first four principal components of the PLS model were fitted.

One of the conclusions in Paper III was that the first and second principal components of the model only explained up to 40% of the variation in the observations. It was thus evident that other parameters, not included in the model, also contributed to the variation between observations. Therefore, besides including the effects of calcium, citrate, urea and their interactions, the model used in this thesis also included storage time and storage temperature.

4 Results and discussion

This chapter presents and discusses the results obtained in this thesis, beginning with results of analyses on variations in raw silo milk and the corresponding fresh UHT milk (Paper I). In section 4.2, the results from the evaluation of UHT milk stored at different temperatures and factors limiting the shelf-life are summarised and discussed (Paper II). The results from the full-factorial study with modified milk are presented and discussed in section 4.3 (Paper III).

4.1 Variations in raw milk and fresh UHT milk

Paper I describes the properties of the raw milk from a commercial dairy plant and the corresponding freshly produced UHT milk, with average values and standard deviations for all traits measured presented in *Table 3*. The average lactose, fat and protein content measured was 4.71, 4.18 and 3.46%, respectively. As expected, processing, including fat standardisation, homogenisation and heating, had a significant impact on some traits, *e.g.* the fat content. Standardisation reduced the fat content from 4.18% in the raw milk to 1.45% in the UHT milk (*Table 3*), which is close to the 1.5% fat content declared on the package. As a consequence of the reduction in fat content, the proportions of lactose and protein increased in the fresh UHT milk. Lindmark-Månsson (2012) and Heck *et al.* (2009), who investigated the composition of Swedish and Dutch raw milk, respectively, reported that the total protein content is highest during January and December. In Paper I, the highest protein content in raw milk was recorded in November and December (>3.50%), while for the rest of the year the protein content varied around 3.45%. The variation in protein content in raw milk can be attributed to diet and stage of lactation.

Unfortunately, the method used to analyse the protein profile of the raw milk did not deliver reliable results with the UHT milk.

Table 3. *Composition and properties investigated in batches of raw milk and the corresponding fresh ultra-high temperature (UHT) milk. Samples were obtained from a commercial dairy plant on a monthly basis during one year, with the exception of August. Values presented are mean and standard deviation (SD)*

	Raw milk		Fresh UHT milk	
	Mean	SD	Mean	SD
Total solids (%)	13.09	0.10	10.58	0.08
Lactose content (%)	4.71	0.03	4.82	0.02
Fat content (%)	4.18	0.05	1.45	0.06
Protein content (%)	3.46	0.04	3.55	0.07
Caseins (%)	81.46	1.38		
Whey proteins (%)	13.19	0.94		
α-lactalbumin (%)	3.15	0.46		
β-lactoglobulin (%)	10.04	0.63		
α_{S1}-casein (%)	28.89	1.04		
α_{S2}-casein (%)	8.00	0.46		
β-casein (%)	40.26	2.29		
κ-casein (%)	4.01	1.76		
Unknown proteins (%)	5.66	1.09		
Calcium (mg/g)	1.21	0.09	1.22	0.08
Potassium (mg/g)	1.39	0.07	1.41	0.10
Sodium (mg/g)	0.40	0.03	0.41	0.02
Magnesium (mg/g)	0.11	0.01	0.11	0.01
Citric acid (%)	0.21	0.01	0.20	0.01
Urea (mg/L)	314	20.0	298	21.9
Total bacteria (cfu/mL)	27900	37000		
Psychrotrophic bacteria (cfu/mL)	1870	3730		
Somatic cell count (cells/mL)	178 000	28 000		
Total proteolysis (leucine equivalents in mM)	40.34	3.17	38.92	2.84
Plasmin activity (U/mL)	3.30	0.52	0	0
Plasminogen-derived activity (U/mL)	91.92	6.19	20.93	3.77
L*	77.76	0.45	79.18	0.81
a*	-0.90	0.28	-1.81	0.12
b*	6.95	0.54	5.40	0.31
Heat coagulation time (min)	19	4	13	2
pH	6.73	0.03	6.72	0.02
Ethanol stability (%)	80	4.5	80	3.9
Freezing point (°C)	-0.533	0.009		

According to EG regulation 853/2004, the raw milk delivered to the dairy should not exceed total bacterial counts of 100 000 cfu/mL or 400 000 SCC/mL. In Paper I, the raw milk was in general found to be of high quality, with an average total bacterial count of 27 900 cfu/mL and 178 000 SCC/mL, *i.e*. far below the maximum permissible levels. However, January had an unexpectedly high number of total bacterial counts, 132 000 cfu/mL, while all other sampling months had total counts of ≤40 000cfu/mL. As UHT milk is considered commercially sterile, bacterial numbers were not evaluated in the UHT milk. However, enzymes of bacterial origin can alter the properties of the milk before processing, leading to a shorter shelf-life of the final product (Stoeckel *et al.*, 2016). The average number of psychrotrophic bacteria was also low, 1870 cfu/mL. However, significantly higher numbers of psychrotrophic bacteria were found in raw milk samples collected in June (5800 cfu/mL) and July (11200 cfu/mL) compared with all the other sampling months (0-756 cfu/mL), giving a high standard deviation for psychrotrophic bacteria (*Table 3*). The cows were outdoors in June-July and most likely subject to higher exposure to bacteria from the environment (soil, pasture, water), which probably explains the peak in psychrotrophic bacteria during the summer months.

The plasmin activity and plasminogen-derived activity in raw milk was on average 3.30 U/mL and 91.92 U/mL, respectively. The UHT process inactivated all plasmin and decreased the plasminogen-derived activity by almost 80%. The results are in agreement with Johansson *et al.* (2017), who reported plasmin and plasminogen-derived activities of 3.63-4.35 U/mL and 88.96-94.64 U/mL, respectively, in raw milk samples from individual cows. The results are also in agreement with Rauh *et al.* (2014b), who observed complete inactivation of plasmin and a 70-90% reduction in plasminogen-derived activity in indirect UHT treatment systems.

In Paper I, the UHT process reduced the HCT from 19±4 to 13±2 min (*Table 3*). Earlier studies recommended a HCT of 20 min at 140 °C for milk for UHT processing (Deeth & Lewis, 2017). The results in Paper I for HCT are in agreement with Gaucher *et al.*, (2008a), who reported the HCT to be 3-18 min in freshly produced commercially available UHT milk.

Some traits remained unaffected by the UHT process in Paper I, including mineral content, pH and ethanol stability. The pH was on average 6.7 in both the raw milk and the fresh UHT milk (*Table 3*). This is in agreement with Deeth and Lewis (2017), who concluded that the UHT process does not affect the pH of fresh UHT milk. According to manufacturers of the UHT processing equipment used in Paper I, raw milk should have a pH above 6.65 to be suitable for UHT processing (Tetra Pak, 2019a). The ethanol stability remained unchanged by the UHT process and was 80% before and after processing (*Table 3*). Chen *et al.* (2015) measured ethanol stability in 25 batches of raw bulk milk and reported an average stability of 93%. The higher ethanol stability, compared to our results, could possibly be due to differences in methodology. In this study samples were checked for visual coagulation after 30 min of incubation at room temperature, whereas Chen *et al.*, (2012) does not indicate if samples were incubate, or as more commonly, checked for coagulation immediately after mixing of the milk sample and ethanol. We found improved reproducibility if reading was not done immediately, incubation >30min did not change the ethanol stability and did not further improve the reproducibility. The ethanol stability values obtained for fresh UHT milk in Paper I are agreement with Gaucher *et al.* (2008a), who reported an ethanol stability range of 75-100%.

Comparing the values obtained in Paper I for raw milk samples collected in northern Sweden (2015) with previous values reported by Lindmark-Månsson *et al.* (2003) and Lindmark-Månsson (2012) for raw milk samples collected throughout Sweden revealed changes in only a few parameters (*Table 4*). Between 2001 and 2009, there was a trend for an increase in calcium content and decreases in citrate and urea contents in Swedish raw milk. The trend for calcium continued between 2009 and 2015 (Paper I), but at a slower rate and the concentration only increased by 2% in that period. In the same period, there was an increase in citrate and urea content of 39% and 11%, respectively (*Table 4*). This shows that the trend for a reduction in citrate and urea contents seen in Swedish raw milk between 2001 and 2009 had not continued to 2015. A noteworthy finding was that the SCC decreased by 20% between 2009 and 2015, reflecting the success of continuous work in Swedish agriculture on improving animal welfare.

Table 4. *Comparison of composition and properties measured in Swedish raw milk in 2009*[1] *and 2015*[2]

	Raw milk 2015		Raw milk 2009		Difference (%)
	Mean	SD	Mean	SD	
Total solids (%)	13.09	0.10	13.14	0.13	0
Lactose content (%)	4.71	0.03	4.73	0.02	0
Fat content (%)	4.18	0.05	4.18	0.11	0
Protein content (%)	3.46	0.04	3.47	0.04	0
Calcium (mg/g)	1.21	0.09	1.19	0.08	2
Potassium (mg/g)	1.39	0.07	1.59	0.07	-13
Sodium (mg/g)	0.40	0.03	0.39	0.01	3
Magnesium (mg/g)	0.11	0.01	0.11	0.00	0
Citric acid (%)	0.21	0.01	0.151	0.146	39
Urea (mg/L)	314	20	282	15	11
Somatic cell count (cells/mL)	178 000	28 000	223 000	27 000	-20
pH	6.73	0.03	6.58	0.04	2
Freezing point (°C)	-0.533	0.009	-0.526	0.004	1

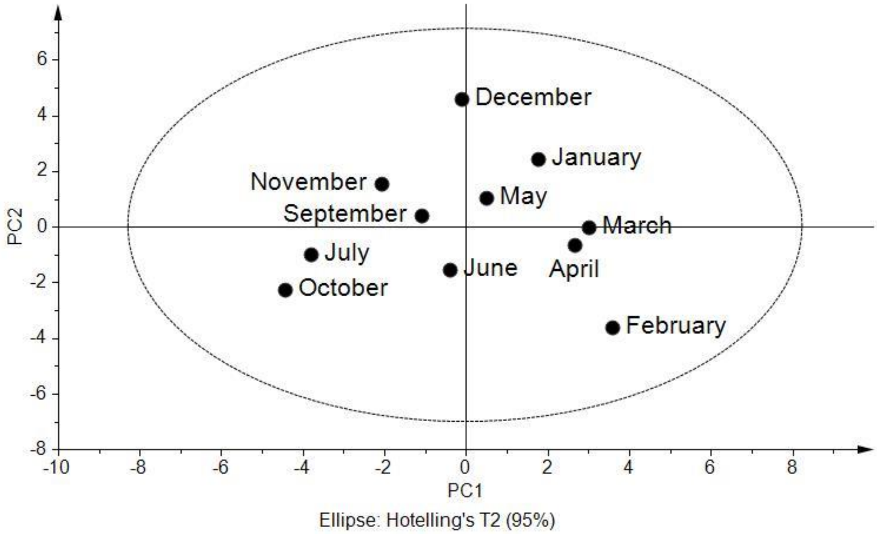
1 Source: Lindmark-Månsson (2012)

2 Source: Paper I

Principal component analysis was used to identify sampling months showing similarities with respect to the total systematic variation in the data. Regarding the variation in Swedish raw milk, the score plot showed only small differences between months (*Figure 5*). As illustrated by the loadings, the major quality traits contributing to differences between months were β-casein, κ-casein, psychrotrophic bacteria, freezing point, urea, calcium and ethanol stability (*Figure 5*). However, the actual variation in some of these quality traits was small (*Table 3*).

Principal component analysis of the data obtained for fresh UHT milk showed a weak tendency for December, January, February and July to deviate from the other months, with calcium, magnesium, lactose and colour (L*a*b*) being the quality traits contributing most to differences between months (*Figure 6*). Comparisons of the actual variation in these parameters generally revealed them to be very constant over the year (*Table 3*).

The levels of different components in raw milk and freshly produced UHT milk observed in Paper I were generally in agreement with observations in previous studies on seasonal variation. However, the PCA results revealed only small variations between sampling months and provided no support for an effect of season on the composition and properties of raw milk or fresh UHT milk. Therefore the effect of season on UHT milk stability can also be expected to be small.



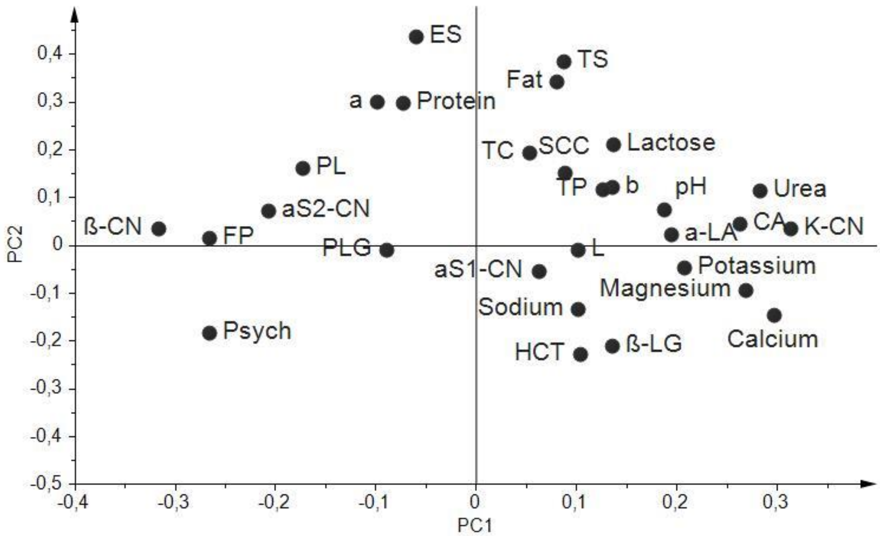


Figure 5. Principal component analysis of quality traits for raw milk collected on a monthly basis during one year, with the exception of August (n = 11). Of the total variation, the first and second principal components (PC1 and PC2) explained 25% and 17%, respectively. Altogether, the five first principal components explained 79% of the variation in the dataset. Abbreviations: αS1-CN = α_{S1}-casein; αS2-CN = α_{S2}-casein; α-LA = α-lactalbumin; β-CN = β-casein; β-LG = β-lactoglobulin; CA = citric acid; κ-CN = κ-casein; ES = ethanol stability; FP = freezing point; HCT = heat coagulation time; PL = plasmin activity; PLG = plasminogen-derived activity; Psych = psychrotrophic bacteria; SCC = somatic cell count; TC = total bacterial count; TP = total proteolysis; TS = total solids.

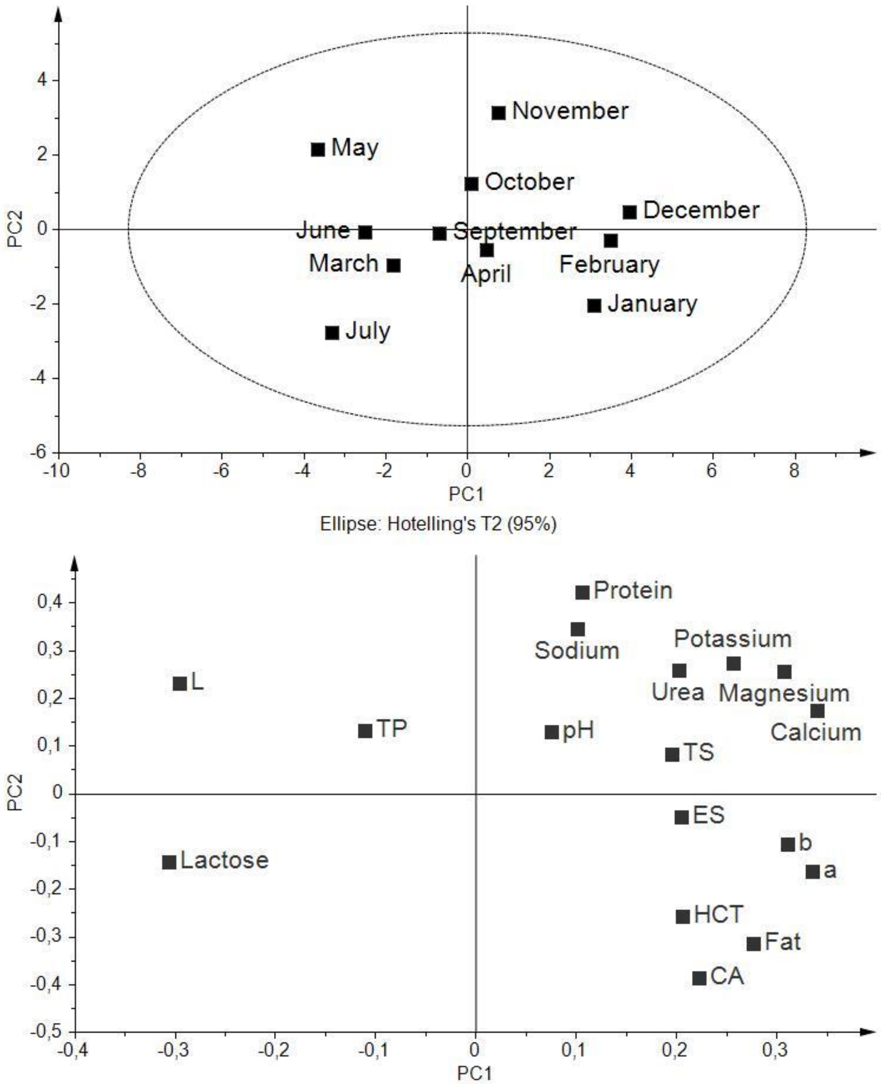


Figure 6. Principal component analysis of quality traits for fresh ultra-high temperature treated milk collected on a monthly basis during one year, with the exception of August (n = 11). Of the total variation, the first and second principal components (PC1 and PC2) explained 43% and 17%, respectively. Altogether, the five first principal components explained 90% of the variation in the dataset. Abbreviations: CA = citric acid; ES = ethanol stability; HCT = heat coagulation time; TP = total proteolysis; TS = total solids.

4.2 Stability during storage

4.2.1 Effect of storage temperature and storage time

Applying PCA for unconditional analysis of all data related to the stored UHT milk produced at the commercial dairy plant revealed that the observations clustered according to storage temperature. This is shown in *Figure 7A*, where the storage temperatures are indicated by colours. Observations made for UHT milk stored at 4 and 20 °C (green and blue) showed similar variation and observations for UHT milk stored at 30 and 37 °C (yellow and red) showed similar variation, but the pattern was different at the lower storage temperatures (*Figure 7A*). These results agree with findings by Gaucher *et al.* (2008b), who stored UHT milk at 4, 20 and 40 °C for up to 26 weeks and found that the stability of UHT milk stored at 4 and 20 °C differed significantly from that of UHT milk stored at 40 °C.

When the observations were colour-coded by storage time rather than storage temperature (*Figure 7B*), there was a clear transition from short-term storage in the upper left corner of the figure to long-term storage towards the right. This is in agreement with findings in several previous studies that chemical and sensory changes in UHT milk are affected by storage time (dos Santos *et al.*, 2018; Jansson *et al.*, 2014; Nielsen *et al.*, 2016).

The first principal component, explaining 57% of the systematic variation in the data, mainly reflected variations in pH and colour. The second principal component, explaining 22% of the systematic variation, mainly reflected variations in fat separation, fat adhesion and sediment formation and in ethanol stability (*Figure 7C*).

When storage time and storage temperature were included as X-variables in the PCA (data not shown), significant correlations (P-value $\leq$0.001) were found for temperature and time with all other X-variables, but storage temperature and time were found to be uncorrelated (P-value >0.1). More specifically, storage temperature was positively correlated with a* and b* values and negatively correlated with L* and pH. Strong positive correlations were found between storage time, fat separation, fat adhesion and sediment formation, and a negative correlation between time and HCT (*Table 5*).

Table 5. *Pearson correlation coefficients for ultra-high temperature treated milk produced at a commercial dairy plant, stored 0-52 weeks at 4, 20, 30 or 37 °C. Storage temperature and storage time correlated significantly with all parameters (P-value <0.001). Abbreviation: ES = ethanol stability; HCT = heat coagulation time; Temp = storage temperature*

	Taste	Fat separation	Fat adhesion	Sedi-ment	L*	a*	b*	HCT	pH	ES
Temp	0.206	0.371	0.538	0.172	**-0.566**	**0.643**	**0.645**	-0.363	**-0.704**	0.553
Time	0.425	**0.709**	**0.695**	**0.885**	-0.561	0.305	0.373	**-0.680**	-0.498	-0.281

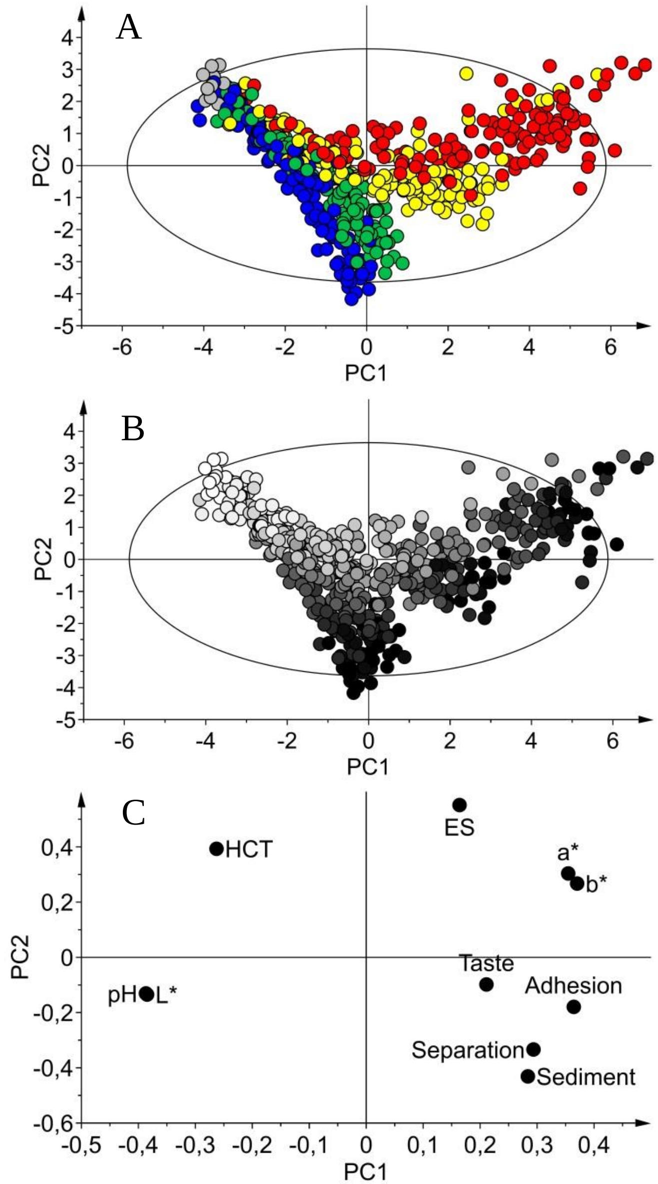


Figure 7. Principal component analysis of quality traits for stored ultra-high temperature treated milk, illustrating differences in stability between samples stored at different temperatures and for different storage times. In score plot (A), the colours represent storage temperatures (grey fresh UHT milk, blue 4 °C, green 20 °C, yellow 30 °C, red 37 °C). In score plot (B), the observations are in gradations from white to black representing storage from 0 to 52 weeks. The systematic variation in the data was explained to 57% and 22% by the first and second principal components (PC1 and PC2), respectively. Abbreviations: ES = ethanol stability; HCT = heat coagulation time.

Table 6. *Evaluation of the sensory attributes measured during long-term storage of ultra-high temperature (UHT) treated milk produced at a commercial dairy plant and stored at 4, 20, 30 or 37 °C for up to 52 weeks. Values represent averages of all sampling months (n = 11) and correspond to storage time in weeks at which the UHT milk was considered no longer acceptable to consumers*

Sensory attribute	Definition	Scale	Threshold for no longer acceptable for consumption	Storage temperature			
				4 °C	20 °C	30 °C	37 °C
Taste	Perceived taste	1 = no deviation 2 = small deviation 3 = large deviation	≥2	39±13 w	33±10 w	29±7 w	23±5 w
Fat separation	Perceived thickness of the fat layer on the surface	0 = no separation 1 = waves 2 = covered 3 = lumps/clots	3	>52 w	>52 w	>52 w	>52 w
Fat adhesion	Thickness and size of the fat layer adhering to the inside of the package after the milk was poured out, compared with reference photos	0 = no fat adhesion 1 = small 2 = medium 3 = large 4 = large and thick	≥3.5	51±3 w	48±10 w	35±11 w	27±6 w
Sediment formation	Size of the sediment layer adhering to the bottom of the package after the milk was poured out, compared with reference photos	0-100%	>45%	35±5 w	31±5 w	23±4 w	21±4 w
Colour	Measured colour	L*, a* and b* values	L* values <76 a* values >-1 b* values >7	>52 w	>52 w	27±5 w	16±3 w

4.2.2 Shelf-life – stability from a consumer perspective

Shelf-life is the period of time in which food products are stable and viable for consumption. From a consumer perspective, the sensory attributes of the milk, including visual stability, *e.g.* fat separation, fat adhesion to the package, sediment formation, gelation, taste and colour, are of critical importance. Sensory methodologies to assess dairy products range from simple tests performed by untrained individuals to tests performed by trained panels, consumer surveys and chemical analysis (Schiano *et al.*, 2017).

Paper II describes how the quality traits varied during storage and the quality traits that limited the shelf-life at different storage temperatures. The shelf-life of the UHT milk stored at 4 and 20 °C was found to be limited by excessive sediment formation and a deviating taste. When the UHT milk was stored at 30 or 37 °C, the shelf-life was limited by several quality parameters including colour, taste and sediment formation (*Table 6*).

4.2.3 Deviating taste

Proteolysis, lipolysis and oxidation contribute in various ways to formation of compounds affecting the stability of UHT milk during storage and perceived by the consumer as unacceptable off-flavours (Schiano *et al.*, 2017).

In Paper II, from storage week 16 onwards every second batch of UHT milk produced at the commercial dairy plant was subjected to a sensory evaluation. For all storage temperatures, when a deviating taste was detected it was generally described as sweet, cardboard, creamy or watery. For UHT milk stored at 30 and 37 °C, the deviating taste was sometimes also described as acidic or caramel (Paper II). It was found that taste deviations developed earlier as storage temperature increased (*Table 6*). Deviating taste was one of the parameters limiting the shelf-life of UHT milk stored at 4 and 20 °C, and was observed on average from storage weeks 39±13 and 33±10, respectively (*Table 6*). For UHT milk stored at 30 and 37 °C, a deviating taste was generally detected from storage weeks 29±7 and 23±5, respectively. A relatively large standard deviation was observed for perceived taste for UHT milk stored at 4 and 20 °C. This was because the batches from July and September developed a deviating sweet taste already after 20-24 weeks of storage, whereas for all other months tested deviations were found after ≥40 weeks. Besides deviating taste developing exceptionally early during storage of UHT milk produced in July, this was also the sampling month with the highest number of psychrotrophic bacteria. Higher numbers of psychrotrophic bacteria in the raw milk are suggested to be correlated with increased proteolysis and bitter taste

(Deeth & Lewis, 2017). However, the deviating taste reported in Paper II during storage in UHT milk produced in July and September was described as sweet. In milk, a sweet taste originates from available sugars, *e.g.* lactose, galactose and glucose. In contrast, in earlier studies by Jensen *et al.* (2015) and Clare *et al.* (2005), a decline in sweetness during storage of UHT milk was detected and was suggested to be caused by a reduction in availability of free sugars, *e.g.* lactose, galactose and glucose, due to ongoing Maillard reactions. The increase in sweetness in some milk samples in Paper II suggests an increase in available sugars or perhaps a decrease in the level of other taste-active compounds, such as cooked flavour, resulting in a sweeter flavour.

Despite the fact that the milk was de-aerated, a cardboard-like flavour was perceived. This could have originated from the oxidation of phospholipids during long-term storage (Walstra *et al.*, 2006). Lipid oxidation and associated formation of volatile aldehydes and ketones can also contribute to the stale flavour in UHT milk (Mehta, 1980), and has been shown to increase with storage temperature (Perkins *et al.*, 2005).

The creamy and watery perception observed in stored UHT milk in Paper II have previously been reported by Jensen *et al.* (2015), who attributed them to changes in the distribution of fat and protein in milk.

In agreement with the results at warm storage temperatures in Paper II, previous studies have shown that product off-flavours can originate from the Maillard reaction, resulting in caramel- and acidic-flavoured products (Clare *et al.*, 2005). Further, although UHT milk is commercially sterile, it is not possible to completely eliminate all microorganisms. Thermophilic spore-forming bacteria have been isolated from UHT milk stored at temperatures >20 °C and are suggested to cause a flat sour deviating taste (Deeth & Lewis, 2017), possibly explaining the sour taste found for milk stored at 30 and 37 °C in Paper II.

4.2.4 Fat separation, fat adhesion and sediment formation

Fat separation, fat adhesion and sediment formation were strongly positively correlated (*Figure 7C*) and correlated with storage time and, to a less extent, with storage temperature (*Table 5*). No lumps or clots of fat were found and fat separation did not reach an unacceptable level at any storage temperature during the 52 weeks of storage in Paper II (*Table 6*). Fat adhesion values ≥3.5 were regarded as unacceptable product quality and UHT milk stored at 4 and 20 °C reached this level in the very last weeks of storage. In contrast, UHT milk stored at 30 and 37 °C reached this limit after 35±11 and 27±4 weeks of storage, respectively. Due to the low fat content of 1.5% and efficient homogenisation in Paper II, fat separation was not an issue and fat adhesion was only regarded as a minor problem after long-term storage at 30 and 37 °C. Methods for measuring fat separation and adhesion vary, as do the results from earlier studies. Despite an extensive fat rise in UHT milk stored at 15 °C for 20 weeks, Deutsch & Jackson (1970) only recorded a few complaints in their study, indicating high consumer acceptance for fat separation. Hansen *et al.* (1980) observed no fat separation when UHT milk with 3.25% fat was stored at 4 °C for 24 weeks, but detected problematic separation in UHT milk stored at 24 and 40 °C for 12 weeks.

At all storage temperatures tested in Paper II, sediment formation increased during storage. Based on the scoring system used to evaluate sensory traits of the UHT milk in, sediment covering >45% of the bottom of the package limited the shelf-life of UHT milk stored at 4 and 20 °C to 35±5 and 31±5 weeks, respectively (*Table 6*). Initially, more sediment formed at the higher storage temperatures, but from storage week 40 onwards sediment formation was higher in UHT milk stored at 4 °C than at the other storage temperatures (*Figure 8A*). Also Ramsey and Swartzel (1984), who stored UHT milk at 7, 22 and 35 °C for 26 weeks, found that the amount of sediment formed increased with storage temperature. Regardless of storage temperature, it has been suggested that sediment consists of proteins or protein particles of various sizes made up of destabilised κ-casein-depleted micelles formed by the UHT process (Gaur *et al.*, 2018; Malmgren *et al.*, 2017).

Fat separation, fat adhesion and sediment formation are suggested to be explained by Stokes' law. As storage temperature increases, the viscosity decreases and differences in density between fat or protein particles and the serum phase increase, allowing faster movement of particles. This explains the higher fat adhesion and sediment formation observed at higher storage temperatures (Anema, 2019). Aggregation of fat globules or micelles further increases the rate of creaming or sedimentation.

Besides Stokes' law, it has been suggested that other mechanisms contribute to sediment formation at different temperatures (Grewal *et al.*, 2017; Malmgren *et al.*, 2017). A possible explanation for the high sediment formation observed at 4 °C after 36 weeks of storage in Paper II is the driving mechanism for casein micelle disintegration at low temperatures. The decrease in hydrophobic interactions (Walstra *et al.*, 2006) and the solubilisation of calcium phosphate that occur at low temperature (Pyne, 1962) contribute to solubilisation of β-caseins, which can lead to destabilisation and aggregation of casein micelles. The result is the formation of larger, heavy particles that settle at the bottom, following Stokes' law (Deeth & Lewis, 2016).

4.2.5 Gelation

Despite the long storage period, no age gelation was observed during the 52 weeks of storage in Paper II. However, shortly after the storage trial ended, gelation was found in samples that were still kept at 4 °C. It is possible that the extensive sediment formation observed in UHT milk stored at 4 °C reflected a transition from sedimentation to initial age gelation (*Figure 8A*). These findings are partly in agreement with Samel *et al.* (1971), who found gelation in UHT milk stored at 4 °C and no gelation in milk stored at 37 °C for 13 months. Samel *et al.* (1971) also observed gelation in milk stored at 20 and 30 °C for over one year.

At least two types of mechanism, enzymatic and non-enzymatic, are known to cause age gelation (Anema, 2019). Enzymatic gelation takes place in UHT milk within a few months of production, due to breakdown of κ-casein caused by heat-resistant proteinases, often originating from psychrotrophic bacteria in the raw milk (Anema, 2019; Dalgleish, 1992). Non-enzymatic or physico-chemical age gelation is slow and is rarely observed within 12 months of storage (Anema, 2019). It tends to be favoured by low storage temperature (Samel *et al.*, 1971), in agreement with our results. For non-enzymatic gelation, no correlation has been found to SCC in raw milk, pH or the Maillard reaction (Anema, 2019).

4.2.6 Changes in colour, pH and heat coagulation time

Throughout the long-term storage period of 52 weeks, the lightness (L*) of UHT milk stored at 4 °C remained >78, whereas for UHT milk stored at 20, 30 and 37 °C, L* values decreased linearly to 76, 72 and 67, respectively (*Figure 8B*). The UHT milk had a brownish colour from storage weeks 27±5 and 16±3 when stored at 30 and 37 °C, respectively (*Table 6*). This unacceptable colour typically corresponded to L* values <76, in *Figure 8B* indicated by the shaded area. The change in colour was strongly correlated with storage temperature (*Table 5*). The observed changes in L* with storage temperature correspond with findings by Auldist *et al.* (1996), who reported L* values of 77-78 after storage of UHT milk for 6 months at 20 °C. In agreement with findings in Paper II, in a recent study measuring changes in colour in skimmed UHT milk during storage at 10-50 °C for up to 24 weeks, L* values were found to change linearly with storage time (Sunds *et al.*, 2018).

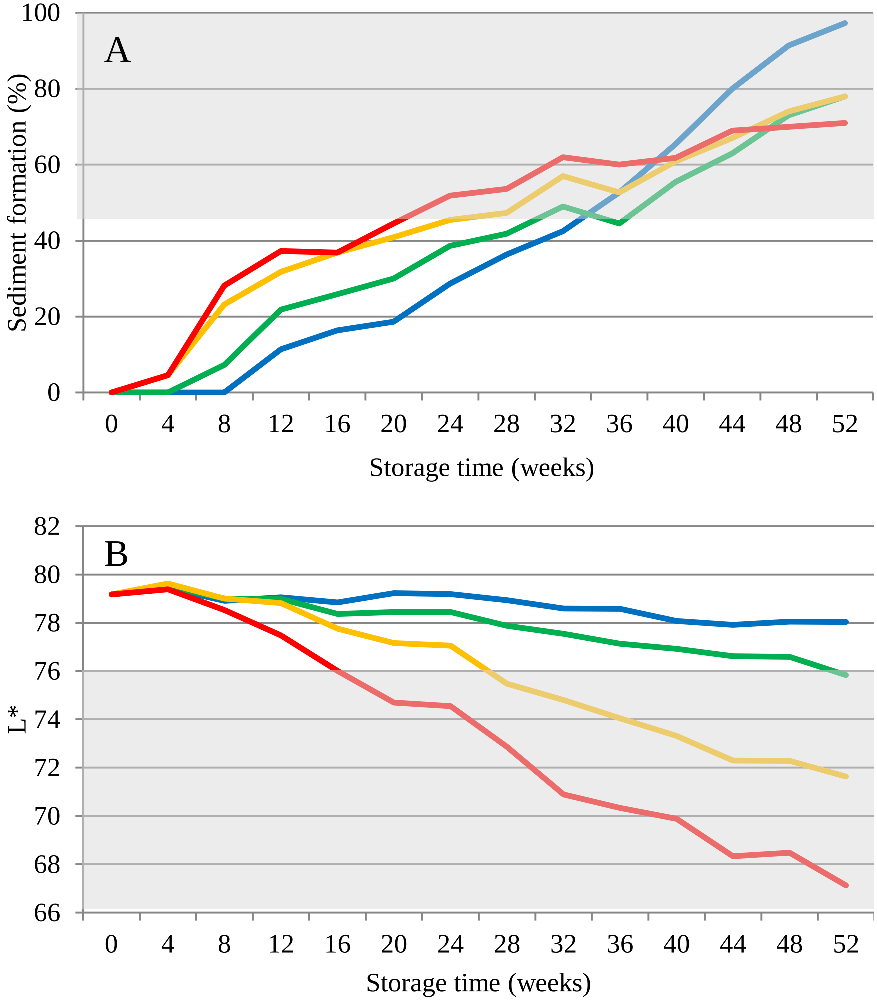


Figure 8. Changes in (A) sediment formation (%) and (B) lightness (L*, dark-light) in ultra-high temperature (UHT) treated milk during storage for 0-52 weeks at 4 °C (blue), 20 °C (green), 30 °C (yellow) and 37 °C (red). Samples with sediment formation >45% and L* values <76 were considered unacceptable to consumers, in the diagram indicated by the shaded areas. Values shown are mean values for 11 batches of UHT milk produced at a commercial dairy plant.

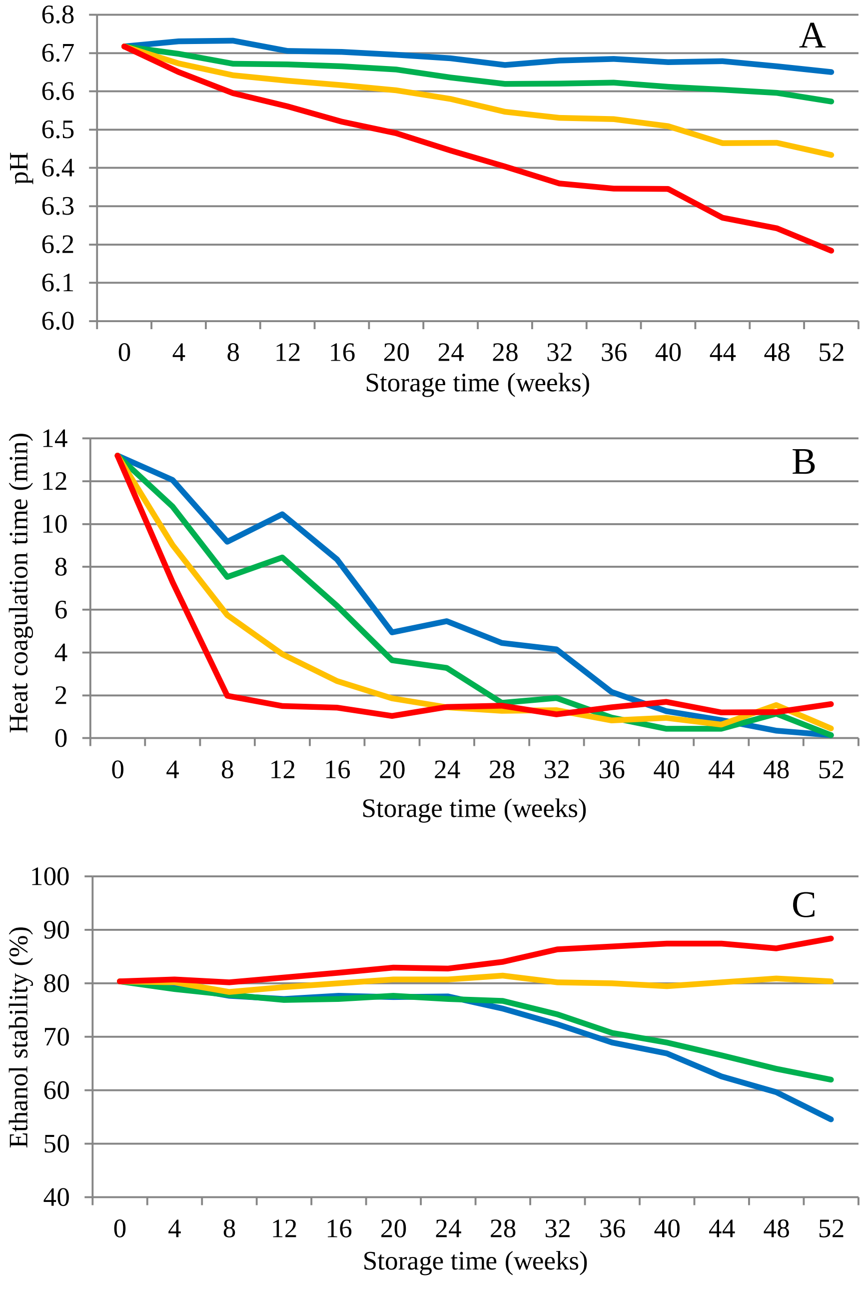


Figure 9. Changes in (A) heat coagulation time, (B) pH and (C) ethanol stability in ultra-high temperature (UHT) treated milk during storage for 0-52 weeks at 4 °C (blue), 20 °C (green), 30 °C (yellow) and 37 °C (red). Values shown are mean values for 11 batches of UHT milk produced at a commercial dairy plant.

Changes in pH and HCT during storage are also likely to be associated with the progress of the Maillard reaction, especially at 30 and 37 °C. The initial pH of 6.7 remained at this level in UHT milk stored at 4 °C during the 52 weeks of storage in Paper II (*Figure 9A*). However, at the highest storage temperature (37 °C), there was a more or less linear decline in pH from storage week 0 to 52, with a final pH of 6.2. In a study by Al-Saadi and Deeth (2008), investigating changes in UHT milk during storage for 12 weeks at 5, 20, 37 and 45 °C, it was found that the initial pH of 6.61 decreased to 6.56, 6.50, 6.43 and 6.35 at these storage temperatures, respectively. Hardham *et al.* (2000) observed that pH decreased by less than 0.1 units in UHT milk stored at 25 °C for 9 months, whereas Gaucher *et al.* (2008b) reported a decrease of 0.5 pH units when UHT milk was stored at 40 °C for 6 months.

In the Maillard reaction, significant amounts of formic acid are formed and are largely responsible for the storage-induced decline in milk pH (van Boekel, 1998). The rate of the Maillard reaction increases with storage temperature, explaining the differences in pH between storage temperatures (Al-Saadi & Deeth, 2008). The decrease in pH can also be attributed to intra-micellar cross-linking reactions or dephosphorylation of caseins resulting in release of protons (Al-Saadi & Deeth, 2008; Holland *et al.*, 2011).

The UHT milk analysed in Paper II had an initial HCT of 13 min (*Figure 9B*). This gradually declined during storage and after 36 weeks of storage at 4 °C the HCT had decreased to <2 min, *i.e.* more or less instantaneous coagulation. For UHT milk stored at 37 °C, the HCT decreased to <2 min already after 8 weeks of storage. As HCT is a subjective determination of the initial point of coagulation, comparison of results from different studies is not always straightforward. Gaucher *et al.* (2008b) measured HCT during storage of UHT milk and found a steeper decline in HCT when milk was stored at higher temperatures (40 °C) compared with 4 and 20 °C, as also found in Paper II. The maximum heat stability of milk is achieved at around pH 6.7 (G. Singh *et al.*, 2007) and small changes in pH can result in major changes in heat stability (Deeth & Lewis, 2016; Jeurnink & de Kruif, 1995; van Boekel *et al.*, 1989). This is because calcium ion activity increases at low pH and the electrostatic repulsion between proteins decreases, promoting aggregation of micelles. Polymerisation-induced coagulation of casein micelles is favoured by pH <6.7, resulting in a shorter HCT (Deeth & Lewis, 2016; van Boekel *et al.*, 1989). This may contribute to the low heat stability of UHT milk stored at elevated temperature, in this thesis 30 and 37 °C.

4.2.7 Ethanol stability

The freshly produced UHT milk had an initial ethanol stability of 80%, decreasing to 55% and 60% after 52 weeks of storage at 4 °C and 20 °C, respectively (*Figure 9C*). Interestingly, in UHT milk stored at 30 °C, ethanol stability did not change during storage and for UHT milk stored at 37 °C there was a tendency for increased ethanol stability during storage. For UHT milk stored at 4 °C, ethanol stability decreased without a corresponding reduction in pH (*Figure 9*). Hence, the effect of pH on ethanol stability observed in raw milk does not occur in stored UHT milk. Samel *et al.* (1971) measured ethanol stability during storage of UHT milk and found a decrease from 96% in fresh UHT milk to 62% when milk was stored at 20 °C for 36 weeks. This is in accordance with the finding in Paper II that ethanol stability decreases at ambient storage temperatures.

The visual coagulation observed in milk that fails the ethanol stability test is believed to originate from the outer hairy κ-casein layer collapsing onto the casein micelle surface and from changes to the internal structure of the micelle, causing reduced steric stabilisation of the casein micelles and consequently aggregation of the micelles (Day *et al.*, 2017; Horne, 2016; Huppertz & de Kruif, 2007). In raw milk, increased intra-molecular cross-linking between κ-casein and β-casein has been shown to increase ethanol stability (Huppertz & de Kruif, 2007). It is reasonable to assume that the same mechanism applies for UHT milk. Holland *et al.* (2011) analysed changes in the protein profile of UHT milk stored for 2 months at 4 °C and found that the α_{s1}-casein fraction remained intact during storage. In contrast, after storage at 28 and 40 °C for 2 months in that study, intra-micellar cross-links between α_{s1}-, α_{s2}- and/or β-casein were observed and suggested to be formed by non-enzymatic deamidation (Holland *et al.*, 2011). In Paper II, intra-micellar cross-links may have contributed to maintaining high ethanol stability in UHT milk stored at 30 and 37 °C, due to stronger electrostatic repulsion between micelles (Horne, 2016).

4.3 Experimental designed study

In Paper III, the milk was modified in order to assess how potential future changes in raw milk composition could affect the stability of UHT milk. The experiment was set up as a full factorial design. The levels of calcium, citrate and urea in milk were increased by 20% compared with the average levels reported in the literature.

4.3.1 Composition and properties of the modified milk before UHT processing

In Paper III, pasteurised and standardised milk was used to produce nine batches of UHT milk. The composition and properties of these batches before UHT processing are summarised in *Table 7*.

Although batches 1-5 and 6-9 were produced on different occasions, only small differences in total solids, lactose, fat, protein, colour and freezing point were found. However, HCT, pH and ethanol stability were noticeably lower in batches with a high calcium content (batches 1, 4, 7 and 8). The pH of batches with an elevated level of calcium was 6.50-6.59 (*Table 7*), *i.e.* below the recommended pH of >6.65 to be suitable for UHT processing (Tetra Pak, 2019a). The ethanol stability in batches with a high calcium content was 38-52%, which is far below the recommended 74% (Shew, 1981). The low pH, low ethanol stability and short HCT found in the milk even before UHT processing indicated that the stability of products with elevated calcium content would be low.

Table 7. *Composition and properties of standardised and pasteurised milk used for ultra-high temperature processing in the full factorial study (Paper III). Batches 1-5 and 6-9 were produced on different occasions. Bold type indicates batches with a high level of calcium. Abbreviations: Ca = calcium, Ci = citrate, Ur = urea, n.a. = not analysed, ref = unmodified reference UHT milk*

Batch	**1**	2	3	**4**	5	6	**7**	**8**	9
	Ca	Ci	Ur	**Ca,Ci,Ur**	ref	ref	**Ca,Ci**	**Ca,Ur**	Ci,Ur
Total solids (%)	**10.70**	10.76	10.70	**10.71**	10.73	10.81	**10.75**	**10.73**	10.81
Lactose (%)	**4.58**	4.56	4.55	**4.57**	4.56	4.62	**4.61**	**4.62**	4.62
Fat (%)	**1.40**	1.42	1.41	**1.40**	1.42	1.46	**1.44**	**1.44**	1.46
Protein (%)	**3.52**	3.57	3.54	**3.54**	3.55	3.54	**3.50**	**3.48**	3.54
L*	**79.64**	77.42	78.08	**77.85**	78.13	78.48	**79.26**	**79.36**	78.51
a*	**-1.74**	-1.73	-1.72	**-1.59**	-1.70	-1.73	**-1.74**	**-1.71**	-1.85
b*	**4.90**	4.89	4.84	**4.84**	4.85	4.47	**4.76**	**4.90**	4.94
Heat coagulation time (min)	**n.a.**	n.a.	n.a.	**n.a.**	n.a.	12	**4**	**<1**	11
pH	**6.52**	6.75	6.77	**6.59**	6.72	6.70	**6.54**	**6.50**	6.77
Ethanol stability (%)	**38**	88	82	**52**	84	86	**48**	**46**	88
Freezing point (°C)	**-0.533**	-0.520	-0.524	**-0.541**	-0.525	-0.531	**-0.540**	**-0.531**	-0.526

4.3.2 Composition and properties of UHT milk made from modified milk

In brief, high calcium content in the milk, and the associated reduction in pH, had a significant negative effect on the stability of UHT milk and was strongly correlated with extensive sediment formation (*Figure 10*). Addition of citrate mainly affected the colour of the UHT milk, suggesting that the amount of citrate added would need to be higher in order to give a significant improvement in stability. The [calcium x citrate] interaction had a small but significant effect on sediment formation and colour, whereas all other interactions and urea had no effect, and thus did not affect the stability of UHT milk.

4.3.3 Effect of calcium, citrate, urea and their interactions

Calcium had a significant effect on all responses tested in Paper III and elevated calcium content, and the corresponding decrease in pH, gave a less stable product compared with the reference UHT milk in multiple ways. It can be assumed that by adding calcium chloride, the calcium equilibrium will be shifted towards the micelle, also increasing ionic calcium levels in serum as well as lowering of pH. As a result, the casein micelles are destabilised, promoting aggregation of micelles and sediment formation (Gaur *et al.*, 2018). In studies by Lewis *et al.* (2011), addition of 4.5 mM calcium chloride to raw milk, corresponding to approximately 15% supplementation of calcium, resulted in large amounts of sediment at a pH of <6.6. However, when the pH of the milk was restored to its original value in that study, sediment formation was reduced almost back to its original value (Lewis *et al.*, 2011). Ramsey and Swartzel (1984) and Malmgren *et al.* (2017) found that the amount of sediment formed depended strongly on increasing storage time and temperature.

Previous studies comparing the addition to raw milk of different calcium salts, *i.e.* calcium chloride, calcium lactate and calcium gluconate, found that the effect on pH, HCT and ethanol stability in the UHT milk varied depending on the type of salt used (Omoarukhe *et al.*, 2010; Singh *et al.*, 2007). For example, those studies found that calcium chloride, which was used in Paper III, had a large negative impact on the stability of UHT milk. This implies that lower levels of calcium chloride or other soluble calcium salts should be used in future studies.

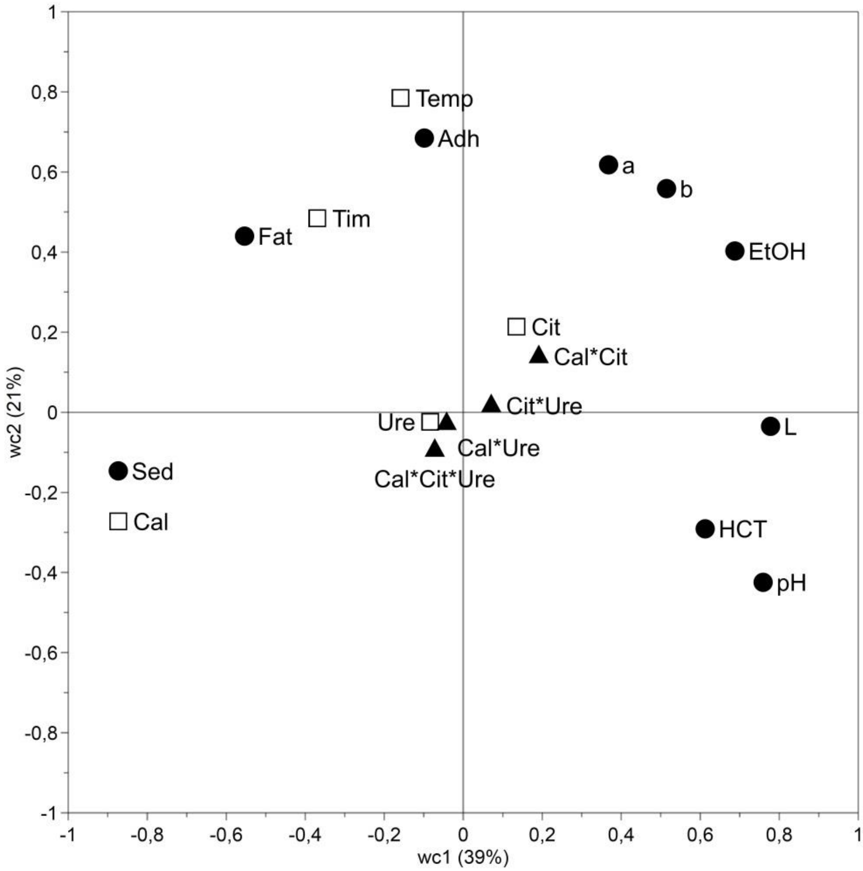


Figure 10. Results of partial least squares (PLS) regression analysis for ultra-high temperature treated milk stored for 0-52 weeks at 4, 20, 30, and 37 °C. □ factors, ▲ interaction terms and ● responses. The systematic variation in the data was explained to 39% and 21% by the first and second principal components, respectively. Abbreviations: a = a*; Adh = fat adhesion; b = b*; Cal = calcium; Cit = citrate; EtOH = ethanol stability; Fat = fat separation; HCT = heat coagulation time; L = L*; Sed = sediment formation.

Comparing the average values of all four samples with elevated citrate content, the factor citrate had a small but significant effect on the colour of the UHT milk (*Figure 10*). Elevating the citrate level by 2 mM did not improve the stability during storage compared with the unmodified reference UHT milk (Paper III). A previous study by Miller and Sommer (1940) showed that addition of 5 mM citrate is required to have an effect on stability. Chen *et al.* (2012) found that addition of 6.4 mM trisodium citrate to goat's milk initially reduced the amount of sediment, whereas addition of 12.8 mM increased sediment formation, suggesting that there is an optimal level of citrate for improved stability of UHT milk. It is therefore possible that the experimental design used in the study, with only two levels of citrate, generated results that are somewhat misleading regarding the stabilising effect of citrate.

In Paper III, urea was located in the centre of the PLS models (*Figure 10*), meaning that this factor had no effect on responses and that the stability during storage was not improved compared with the reference UHT milk. Earlier studies indicate that a level of >7 mM urea is needed to improve stability (Muir & Sweetsur, 1977). In Paper III, urea was added to a final calculated concentration of 5.6 mM which, compared with results from earlier studies, was apparently too low to have an effect on UHT milk stability.

The combination of elevated levels of calcium and citrate resulted in a short delay of a few weeks in sediment formation during storage, but did not prevent heavy sediment formation (Paper III). The actual effect of the [calcium x citrate] interaction on sediment formation could therefore be questioned. All other interactions ([calcium x urea], [citrate x urea], [calcium x citrate x urea]) had very little effect and were located at the centre of the PLS plot (*Figure 10*).

4.3.4 Effect of storage time and storage temperature

Besides the effects of calcium, citrate, urea and their interactions, the model used for *Figure 10* includes storage time and storage temperature. The systematic variation in the first principal component, contributing to 39% of the variation, can be explained by the main effect of calcium on all responses, positively correlated with sediment formation and fat separation, uncorrelated with fat adhesion and negatively correlated with all other responses (*Figure 10*). Inclusion of storage time and temperature improved in particular the variation explained by the second principal component, from 4 to 21% (Paper III). This suggests that storage time and temperature contribute to a large extent to the stability of UHT milk.

4.4 General discussion

Paper I describes the composition and properties of raw milk, and the corresponding fresh UHT milk, collected monthly during one year from a commercial dairy plant located in northern Sweden. The hypothesis was that raw milk differs in composition over the year and that these differences persist in the fresh UHT milk. Paper I specifically examined seasonal variations, comparing the outdoor grazing period with the indoor period. However, the differences in raw milk were smaller than expected, indicating consistent raw milk quality all year round and no seasonality in the composition and properties of the raw milk or the corresponding fresh UHT milk. It is important to have in mind, that this thesis does not cover variation in fatty acid composition of the milk and it is know that the fatty acid composition in milk produced when cows are grazing, will differ compared to milk produced when cows are indoors, by being more unsaturated (Lu *et al.*, 2018). Further in Paper I, the parameters mainly contributing to differences between months were not the same for the raw milk and the fresh UHT milk, indicating that the UHT process has a significant impact on the composition of the resulting product. Overall, the results obtained in Paper I of this thesis suggest that, with modern and well-managed herds, there is very little variation in raw milk composition throughout the year. Thus the effect of season on UHT milk stability can also be expected to be small.

Even though variations in the fresh UHT milk were small, there were still variations in shelf-life between different batches of UHT milk. These variations in shelf-life cannot be fully explained by variations in parameters measured in this thesis. Other parameters evidently play a role, *e.g.* it is known that the fat composition of raw milk varies significantly between months (Lu *et al.*, 2018) and the fat composition affects the taste (Deeth & Lewis, 2017). It is also possible that small variations in processing, *e.g.* slightly higher temperature or longer holding time, give a higher heat load that will inactivates more enzymes but also increases the Maillard reaction, affecting the shelf-life of UHT milk (McMahon, 1996).

The duration of cold storage, on the farm or at the dairy plant, before heat treatment is also known to affect the stability of UHT milk (Deeth & Lewis, 2017). For example, a long period of cold storage gives the enzymes in the milk, mainly originating from psychrotrophic microorganisms, more time to affect the properties of the milk, possibly contributing to shorter shelf-life (Deeth & Lewis, 2017).

The sensory attributes measured in this thesis were only correlated with ethanol stability to a limited extent, indicating that the shelf-life of UHT milk is poorly explained by ethanol stability in the milk.

Surprisingly, during storage of UHT milk no bitter-tasting samples were found and age gelation happened much later than expected, *i.e.* after the period of study (>52 weeks), suggesting very low enzymatic activity in the UHT milk.

For the study examining changes during storage of UHT milk (Paper II), the starting hypothesis was that the stability of UHT milk is significantly affected by storage temperature and storage time. The results obtained in Paper II confirmed this hypothesis, with all parameters measured found to be significantly affected by storage temperature and time. For most parameters, an increase in storage temperature led to a decrease in stability. The main exception was ethanol stability, which remained high in UHT milk stored at higher temperatures.

In addition to use of pH, HCT and ethanol stability to assess the suitability of raw milk for UHT processing, in this thesis these parameters were used as indicators when evaluating changes in UHT milk stability during storage (Papers II and III). The results showed that correlations for pH-HCT and pH-ethanol stability based on measurements on raw milk are not applicable to UHT milk. For instance, the decrease in pH given by the Maillard reaction, seen at the higher storage temperatures, affected the HCT. Van Boekel *et al.* (1989) reported that HCT is very sensitive to changes in pH and at pH <6.6 inter-micellar cross-linking is promoted, in our study observed as a drastic decrease in HCT. A lower pH is also suggested to give a lower ethanol stability. However, our results showed that ethanol stability of UHT milk is not mainly affected by changes in pH. Instead other processes are suggested to be more important for the ethanol stability, including intra-micellar cross-linking that *e.g.* could be formed by the Maillard reaction or deamidation. It is evident that the chemical and physical processes occurring simultaneously during storage of UHT milk are many and complex and how the reactions interact, affecting the stability, are not yet fully understood.

In the full-factorial experiment (Paper III), the hypothesis was that an elevated level of calcium would decrease the stability of UHT milk and that elevated levels of citrate and urea would increase the stability, but also that the negative effect of calcium would be compensated for by buffering or stabilising effects of citrate and urea. The strong instant negative effect of elevated level of calcium was not expected and it was surprising that citrate and urea did not improve the stability compared with the unmodified UHT milk. The study showed the effect of modifying the raw milk composition and, more importantly, the strong effect of storage time and temperature on the stability of UHT milk during storage.

In stability testing of products with a long shelf-life, it can be tempting to speed up the test time by storing the product at a temperature higher than normally recommended. Many chemical reactions will proceed faster at higher temperature according to simple kinetic models, thus decreasing the time until the product is no longer acceptable for consumption. However, as shown in this thesis, different sensory attributes and mechanisms limit the shelf-life of UHT milk at different storage temperatures. It is therefore recommended that acceleration of experimental testing by increasing the storage temperature should be used with caution.

5 Conclusions

- Principal component analysis revealed some differences throughout the calendar year in the composition of raw milk and of the corresponding fresh UHT milk. However, the variations in parameters measured in this thesis were small and were not found to be associated with season.
- Storage temperature and storage time had a significant impact on the stability and shelf-life of UHT milk.
- The shelf-life of UHT milk was longer at cold or ambient storage temperatures. At these temperatures, the shelf-life was limited to 31-35 weeks by excessive sediment formation, possibly due to mechanisms such as protein aggregation, followed quickly by a deviating taste previously reported to correlate with enzymatic activity or oxidation.
- Storage temperatures ≥30 °C considerably decreased the shelf-life of UHT milk, to 16-23 weeks. Shelf-life at these temperatures was limited by deviating colour and taste, possibly attributable to the Maillard reaction, and sediment formation due to protein aggregation.
- An elevated level of calcium, and the associated reduction in pH upon addition of calcium chloride to milk, had a significant negative effect on the stability of the corresponding UHT milk and was strongly correlated with instant extensive formation of sediment.
- Citrate addition to milk and the interaction between calcium and citrate addition mainly affected the colour of the UHT milk. However, when milk with high citrate, or high calcium in combination with high citrate content were compared with reference UHT milk, these factors only contributed to a limited extent contributed to improved stability during storage. This suggests that other concentrations of citrate than that used in this thesis are needed to give a significant improvement in the stability of UHT milk.
- Elevating the level of urea in the milk did not affect the stability of UHT milk. This suggests that higher concentrations of urea than that used in this thesis are required to have an effect on the stability of UHT milk.

6 Future research

In this thesis, which studied the impact of natural and experimentally introduced variations in raw milk composition on UHT milk stability, the UHT process was unaltered throughout the trial period. Other studies have shown that the raw milk and the process itself have significant effects on the stability of the resulting UHT milk. Therefore it would be relevant to conduct detailed studies that can distinguish the respective effects of the raw milk and the process.

Three parameters were included in the full factorial experiment in this thesis, namely calcium, citrate and urea. Swedish data on raw milk quality indicate that the levels of these parameters have changed significantly over the past decade, with calcium content increasing and citrate and urea decreasing (Lindmark-Månsson, 2012). Based on the results in this thesis, this would have negative implications for the stability of UHT milk. However, other parameters in raw milk in Sweden, including pH and fatty acid composition, have also changed significantly in recent decades. In future studies, it would therefore be interesting to examine how other parameters affect the stability of UHT milk during long-term storage. The fatty acid composition contributes to the sensory attributes (taste and creaming) and variations in fatty acid composition of the raw milk could have an impact on the sensory attributes of the UHT milk. It would also be interesting to obtain more recent data on Swedish raw milk composition. Over the past decade, advances in animal breeding and changes in feeding and management systems may have caused further changes in raw milk composition.

In the full factorial experiment described in this thesis, calcium chloride was used to increase the calcium content, but this also led to a reduction in pH. In future studies, pH should be kept constant, *e.g*. by adjusting the pH back to its original value after addition of calcium chloride. In Paper III, pH was measured as a response variable, but the results showed that variations in pH significantly affected the stability of the UHT milk. A weakness of the full factorial experiment was that only two levels (natural and elevated) of the tested parameters were considered. In future studies, it would be interesting to study additional elevated levels of calcium, and possibly also reduced levels, so as to identify upper and lower recommended levels for raw milk to be used for UHT processing.

To fulfil the goal of the National Food Strategy for Sweden (*Livsmedels-strategin*) on increased profitability and improved competitiveness in food production, resources for research on production systems must be provided (Prop. 2016/17:104). A sustainable and competitive food sector includes economic sustainability, so research that links biological aspects with financial aspects of food production is needed. This could examine *e.g.* whether it would be profitable to do more tests on the raw milk and use milk of different quality for different dairy products. The calcium content and also free fatty acid content of the raw milk should perhaps be analysed more regularly, as both have been shown to vary over time and affect the stability and shelf-life of the resulting product. Another question for future research is whether it is necessary to use UHT treatment or whether similar stability could be achieved by using raw milk of high quality with extended shelf-life processing. A milder heat treatment like extended shelf-life (ESL) processing would reduce the energy requirement for heating. In addition, ESL-treated milk has sensory properties similar to those of pasteurised milk.

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Popular science summary

Better knowledge of how the composition of the unprocessed raw milk affects the quality of milk products and how the storage temperature affects the best-before date, will hopefully allow the dairy industry extend the shelf-life of long-life milk with maintained product quality.

For consumers in general, quality can be defined by consistency in appearance and taste of a product. Meeting this demand can be a challenge for the dairy industry, as it is known that the composition of the raw milk varies over the year, affecting the quality of dairy products. The natural variations in the raw milk are believed to be partly due to that Swedish cows are grazing in the summer, *i.e.* they eat a different feed compared to the rest of the year. Other factors affecting the milk's composition include the age of the cow, breed and bacteria in the milk.

In this PhD project, we studied the natural variations in raw milk from northern Sweden. We also investigated how the composition and properties of the raw milk affected the best-before date of long-life milk, so-called UHT (ultra-high temperature) treated milk, and how the storage temperature affected the best-before date.

UHT milk is produced by heating milk to at least 135 °C for a few seconds. The combination of the heating method and the way the milk is packaged gives a product that is basically free of bacteria and thus lasts for several months when stored at ambient temperature, which is convenient in cases when it is not possible to store the milk refrigerated. A disadvantage of the high heating temperature is that the milk can get a slightly cooked taste, which is not appreciated by all consumers.

In a previous study, investigating the composition of Swedish milk, it was found that the levels of calcium had increased, while citrate and urea had decreased. Based on what is known today, these changes together could have a negative effect on the shelf-life of UHT milk. Therefore, in an experimental study performed at Tetra Pak's Product Development Centre in Lund we added calcium, citrate and urea, in different combinations, to milk to study the effect on the shelf-life of the UHT milk by varying these components. The modified milk was UHT treated and stored at 4, 20, 30 and 37 °C for up to one year. Samples were analysed each month to study how the quality of the UHT milk changed during long-term storage. Our results showed that high level of calcium significantly shortened the best-before date, while the elevated levels of citrate and urea, used in this study, had no effect on the stability.

To study the natural variation in raw milk, we collected samples of unprocessed milk from Norrmejerier's facility in Luleå every month for one year. Samples of the resulting UHT milk were also collected. Both the raw milk and the fresh UHT milk were analysed for composition and different properties, including protein, fat and lactose content, total number of bacteria and the heat stability of the milk. Subsequently, the UHT milk was stored at 4, 20, 30 and 37 °C for up to one year. Every month, milk samples were analysed with respect to taste and appearance, but also other quality attributes such as pH and heat stability were analysed.

Our results showed that the raw milk had a high and consistent quality all year round. Because of the grazing season, we had expected greater differences in milk composition between the summer months and the rest of the year. Although the raw milk kept a consistent quality, the shelf-life of the resulting UHT milk varied between production months. For some production months, with time the milk got a deviating taste, for other months a deviating colour. The fact that some batches of UHT milk had longer, other shorter shelf-life cannot be explained by the parameters we analysed in the raw milk. Instead, variations in milk fat composition or small variations in the process may be the reasons for the variation in stability of the UHT milk over the year.

UHT milk that was stored cold or at room temperature had the longest shelf-life, on average over 7 months, which is several months longer than the best-before date indicated on the package. However, when the storage temperature was raised to above normal room temperature, the shelf-life was considerably shortened due to the milk got brownish, and in this case the actual shelf-life corresponded to the best-before date on the package.

In summary, consumers who buy UHT milk are advised to store the product refrigerated or at room temperature as this will provide the longest shelf-life. However, an opened package must be stored refrigerated. We recommend that dairies, in addition to their daily quality tests, regularly follow the long-term changes in the raw milk to ensure that the milk composition will provide good prerequisites for the production of sustainable dairy products.

Populärvetenskaplig sammanfattning

Med bättre kunskap om hur mjölkråvarans sammansättning påverkar kvaliteten på mjölkprodukter och hur förvaringstemperaturen påverkar bäst-före-datumet är förhoppningen att mejerierna kan förlänga hållbarheten på mjölk med extra lång hållbarhet utan att tumma på dess kvalitet.

För konsumenter i allmänhet kan hög kvalitet vara att en produkt smakar och ser likadan ut året om. Att uppfylla detta kan vara en utmaning för mejerierna, då det är känt att mjölkråvarans sammansättning varierar över året, vilket kan påverka mejeriprodukternas kvalitet. Variationerna i råvaran tros bland annat bero på att svenska kor betar på sommaren och då äter ett annat foder jämfört med resten av året. Andra faktorer som påverkar mjölkens sammansättning är kons ålder, ras och bakterierna i mjölken.

I detta doktorandprojekt studerades de naturliga variationerna i norrländsk mjölkråvara. Vi undersökte också hur mjölkråvarans sammansättning och egenskaper påverkar bäst-före-datumet för mjölk med extra lång hållbarhet, så kallad UHT (ultrahög temperatur) behandlad mjölk och hur lagrings-temperaturen påverkar UHT-mjölkens bäst-före-datum.

UHT-mjölk tillverkas genom att mjölk hettas upp till minst 135 °C under ett par sekunder. Kombinationen av upphettningsmetod och sättet som mjölken förpackas gör mjölken i princip fri från bakterier och därmed håller mjölken i flera månader när den förvaras i rumstemperatur, vilket är praktiskt om man inte har möjlighet till kylförvaring. En nackdel med den höga uppvärningen är att mjölken kan få en något kok smak, som inte uppskattas av alla.

I en tidigare undersökning av den svenska mjölkens sammansättning konstaterades att nivåerna av kalcium hade ökat, medan citrat och urea hade minskat. Utifrån det man idag känner till, skulle dessa förändringar sammantaget kunna leda till att UHT-mjölkens hållbarhet minskar. Därför genomfördes en experimentell studie vid Tetra Paks produkt-utvecklingscenter i Lund för att studera effekten av att variera dessa komponenter i mjölkråvaran. Kalcium, citrat och urea i olika kombinationer tillsattes till obehandlad mjölk som UHT-behandlades och förvarades i 4, 20, 30 och 37 °C i upp till ett år. Prover togs ut varje månad för att studera hur UHT-mjölken förändrades vid långtidsförvaring. Våra resultat visade att höga nivåer av kalcium avsevärt förkortade UHT-mjölkens bäst-före-datum medan de högre nivåer av citrat och urea som användes i denna studie inte hade någon effekt på hållbarheten.

För att studera den naturliga variationen i mjölkråvaran samlade vi varje månad under ett år in prover på obehandlad mjölk från Norrmejeriers anläggning i Luleå. Även prover från den resulterande UHT-mjölken samlades in. Såväl den obehandlade råvaran som den färska UHT-mjölken analyserades med avseende på sammansättning och olika egenskaper. Bland annat mättes protein-, fett- och laktoshalt, det totala antalet bakterier, celltal och mjölkens värmestabilitet. Därefter förvarades UHT-mjölken i olika temperaturer upp till ett år. Mjölkprover togs ut varje månad och smak och utseende, samt andra kvalitetegenskaper, t.ex. pH och värmestabilitet analyserades.

Våra resultat visade att mjölkråvaran höll en hög och jämn kvalitet året runt. Med anledning av den lagstadgade betessäsongen hade vi förväntat oss större skillnader i mjölkens sammansättning mellan sommarmånaderna och övriga året. Trots att råvaran höll en jämn kvalitet såg vi att UHT-mjölken varierade i hållbarhet mellan olika produktionsmånader. Vissa månader fick mjölken en avvikande smak, andra månader en avvikande färg. Att vissa batcher av UHT-mjölk hade längre, andra kortare hållbarhet, kan inte förklaras med de parametrar som vi analyserade i mjölkråvaran. Istället kan variationer i mjölkfettets sammansättning eller små variationer i processen ha orsakat att UHT-mjölkens hållbarhet varierade över året.

UHT-mjölk som förvarades kallt eller i rumstemperatur höll längst, i genomsnitt över 7 månader, vilket är flera månader längre än bäst-föredatumet som angavs på förpackningen. När förvaringstemperaturen höjdes till över normal rumstemperatur förkortades dock hållbarheten kraftigt på grund av att mjölken blev brunaktig, och den faktiska hållbarheten motsvarade i detta fall förpackningens bäst-före-datum.

Sammanfattningsvis rekommenderas konsumeter som köper UHT-mjölk att förvara den kallt eller i rumstemperatur då detta ger längst hållbarhet. En öppnad förpackning måste dock förvaras kyld. Till mejerierna rekommenderas att, utöver de dagliga kvalitetstester som görs, regelbundet följa den långsiktiga förändringen i mjölkråvaran för att säkerställa att mjölkens sammansättning ger goda förutsättningar för produktion av hållbara mejeriprodukter.

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